Impaired c-src activation and motility defects in PEA3-null fibroblasts

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Abstract

Null mutations in the pea3 allele compromise the capacity of mammary tumors to metastasize in MMTV-Neu/ErbB2/HER2 transgenic mice, indicating a motility defect in PEA3-null cells. Cellular and biochemical analyses of established PEA3-null fibroblasts show impaired motility and aberrant localization of adhesion proteins in spreading cells. Our results show that PEA3−/− cells express normal levels of key adhesion components, but that spreading PEA3-null cells fail to activate c-src and to downregulate phospho-FAK(Y397), suggesting that focal adhesion signaling is impaired. Supporting this, biochemical analysis revealed that adhesion complex-associated proteins such as p130Cas failed to undergo tyrosine phosphorylation and dissociated from the adhesion complex with delayed kinetics. Overall our data show that the motility defects observed in PEA3-null cells are due to altered adhesion signaling.

1. Introduction

PEA3/E1AF/ETV4 is the founding member of a subfamily of ETS transcription factors implicated in the gene regulation of membrane receptors, growth factors and extracellular matrix metalloproteinases [1,2]. PEA3 proteins have been shown to be overexpressed in the vast majority of human breast and ovarian tumors and in nearly all of the human epidermal growth factor receptor 2 (HER2/Neu/ErbB2)-positive subclass of breast tumors [3-6]. Interestingly, null mutations in the pea3 allele compromise the capacity of mammary tumors to metastasize in MMTV-Neu/HER2/ErbB2 transgenic models of breast cancer [7-9]. In contrast, overexpression of PEA3 in MCF7 human breast tumor cells increases their metastatic potential [10], whereas PEA3 antisense RNA reduces the invasiveness of human tumor cells [11]. These results strongly suggest a role for PEA3 in the regulation of target genes important for the control of cell motility.

Cell adhesion and migration involves the dynamic assembly and disassembly of adhesion components at the leading edge of migrating cells. Stimulation of cell adhesion and migration induces the formation of an integrin-FAK-src complex that is required for the recruitment and activation of a number of adaptor molecules leading to focal adhesion turnover and migration [12-14]. Indeed, FAK-null cells assemble large and stable adhesion complexes leading to migratory deficits [15]. Similarly, a FAK mutant at tyrosine 397, deficient for c-src binding, fails to induce focal adhesion disassembly in FAK-deficient fibroblasts [16,17]. Supporting this, src-family kinase-deficient cells or expression of kinase inactive v-src in fibroblasts display larger focal adhesions that fail to disassemble [18,19].

The reduced capacity of PEA3-null breast tumors to metastasize suggests that PEA3 regulates a subset of genes important for motility and invasion. To gain insight into the migratory defect of PEA3−/− cells, we have characterized the subcellular distribution of adhesion markers and the association of adapter proteins with the FAK-src complex in PEA3-null fibroblasts. Our results show that PEA3−/− cells exhibit migration deficiencies in Boyden chamber assays. Although PEA3−/− cells display normal distribution of focal adhesions, re-plating on fibronectin (FN) shows reduced c-src activation and delayed focal complex disassembly. Our results suggest that a subset of PEA3 target genes encodes proteins implicated in c-src regulation and focal adhesion signaling.

2. Materials and methods

2.1. Cell lines and cell culture

The pea3-null mouse embryonic fibroblast cell lines, PEA3(−/−), 1, 2 and 3, as well as the PEA3(+/−) cells stably expressing PEA3 cDNA and the wild-type cells (WT) were derived from PEA3(−/−) E13.5 embryos [7] and established following the 3T3 protocol. All cell lines

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were maintained at 37 °C in a humidified atmosphere containing 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM medium, Bio-Whittaker) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin.

For replating assays, subconfluent cultures were serum-starved in 0.25% FBS–DMEM medium for 24 h and harvested by trypsin-EDTA treatment as described previously [15]. The trypsin was inactivated using soybean trypsin inhibitor (0.5 mg/ml) (Sigma) and the cells were collected by centrifugation and resuspended in 0.1% bovine serum albumin–DMEM (BSA–DMEM) (w/v) medium. After 1 h at 37 °C in suspension, the cells were plated onto FN or Poly-l-Lysine pre-coated coverslips. The coverslips were pre-coated with FN (10 μg/ml, Sigma) or Poly-l-Lysine (10 μg/ml, Sigma) in PBS overnight at 4 °C, rinsed with PBS, and warmed to 37 °C for 1 h prior to replating.

For wound healing assays, exponentially growing cells were plated onto FN-coated coverslips in complete growth medium and incubated overnight at 37 °C. The next day, the monolayer of cells was washed twice with PBS and once with 0.1% BSA–DMEM medium. The wound healing assay was initiated by manually scratching the monolayer of cells with a pipette tip and allowing migration in 0.1% BSA–DMEM medium at 37 °C for 6 h. Migration was quantitated using Millicell Boyden chambers (Millipore) as described previously [20]. The lower side of the polycarbonate membranes were pre-coated with BSA (1% w/v) or FN (10 μg/ml) overnight at 4 °C, rinsed with PBS, and warmed to 37 °C for 1 h prior to the initiation of migration. Serum-starved cells collected and resuspended in 0.1% BSA–DMEM medium were added to the upper chamber to initiate cell migration. Following migration, 4 h at 37 °C, both chambers were rinsed with PBS and the lower side of the membrane was fixed in 4% paraformaldehyde (PFA) and stained with DAPI. The remaining cells in the upper chamber were removed using a cotton tip applicator. The number of migrated cells was determined by counting the DAPI stained cells (cells/field using a ×20 objective) on the underside of the membrane. Cells were enumerated from 5 random fields and averaged.

2.2. Immunofluorescence

Coverslips were fixed in 4% PFA for 10 min at room temperature, washed in PBS, and blocked with 1% BSA or 200 μg/ml ChromaPure Goat IgG (Jackson ImmunoResearch Laboratories). Focal adhesions were visualized with a FAK polyclonal (Santa Cruz), a paxillin monoclonal (BD Biosciences) and a mouse anti-vinculin monoclonal antibody (clone VIN11-5, Sigma) in conjunction with fluorescein isothiocyanate (FITC)-labeled secondary antibodies. TRITC-conjugated phalloidin was used to detect actin stress fibers. Samples were visualized on a Zeiss Axioskop100 epifluorescence microscope equipped with appropriate filters and photographed with a digital camera (Sony Corporation) using the Northern Eclipse software package.

2.3. Immunoprecipitations and western blots

Cells were rinsed in PBS and protein extracts were made in modified RIPA buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Nonidet P-40, phosphatase inhibitors (10 mM NaF, 10 mM β-glycerophosphate and 250 mM Na ortho-vanadate) and protease inhibitors (Sigma inhibitor cocktails). For western analysis, β1 integrin, PTEN, RhoA, Rac1 and Cdc42 antibodies were from SantaCruz. Anti-vinculin and anti-c-src were obtained from Sigma and anti-paxillin antibodies were purchased from BD Biosciences. For immunoprecipitations, 400 μg of total cell lysate was immunoprecipitated using 2 μg of anti-FAK rabbit polyclonal antibody (clone 556368, BD Pharmingen) or anti-paxillin mouse monoclonal antibody (clone 610051, BD Biosciences) and 20 μl of protein A-Sepharose (Amersham Biosciences) for 4 h at 4°C. Immunoprecipitates were washed three times with NETN (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, and 0.1% (v/v) Nonidet P-40) and eluted with 4× SDS sample buffer (containing 200 mM Tris–HCl pH6.8; 400 mM DTT; 8% (w/v) SDS; 0.4% (w/v) bromophenol blue and 40% (v/v) glycerol). Samples were fractionated on 8% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF). Membranes were then probed with anti-p130Cas monoclonal antibody (P27820, Transduction Laboratories), anti-phospho-p130Cas antibody (Tyr249; 4014S, Cell Signaling) or anti-c-src (Sigma). FAK and c-src activation was determined by western blotting of whole cell lysates with rabbit polyclonal anti-FAK phosphospecific antibodies for phospho-tyrosine residues 397 (pY397) and anti-c-src phosphotyrosine 416 specific antibody (c-src pY416; Upstate Biotechnology), respectively. To evaluate the efficiency of the immunoprecipitation, PVDF membranes were stripped using Re-Blot Plus (Chemicon International) and re-probed with anti-FAK or anti-paxillin accordingly. Reactive proteins were detected by enhanced chemiluminescence (PerkinElmer Life Sciences) using a goat anti-rabbit or anti-mouse horseradish peroxidase-labeled secondary antibody and visualized using autoradiography.
3. Results

3.1. Impaired motility in PEA3-null fibroblasts

Previous studies have shown that PEA3 is required for the invasive potential of breast tumors in vivo and in vitro [7–11]. To investigate whether the reduced invasion potential of PEA3-null tumors was due in part to a motility defect, three independent PEA3(−/−) fibroblast cell lines and a PEA3 reconstituted line were analyzed. All MEF cell lines have been previously characterized for PEA3 deletion and expression [21]. The cultures were initially subjected to wound healing and Boyden chamber haptotactic assays. Following scratch wounding of the confluent monolayers (Fig. 1A), wildtype (WT) and PEA3-reconstituted cells nearly closed the wound within 6 h whereas PEA3-null cells displayed a 40–50% closure. Interestingly, PEA3(−/−) cells did not seem to form filopodial structures or extensions into the wound as did wildtype cells (Fig. 1C and D). To quantitate the observed migratory defect, we performed FN haptotactic assays. All three PEA3(−/−) cell lines displayed a relative 4-fold decrease in migration on fibronectin when compared to WT or PEA3-reconstituted cells (Fig. 1E–H). No differences were observed in random migration on BSA. Together, these results suggest that PEA3 target gene products regulate important aspects of cell migration.

3.2. Failure to activate c-src leads to impaired focal adhesion disassembly in PEA3(−/−) cells

To further investigate the migration defect in PEA3(−/−) cells, we first examined the expression level of key adhesion complex proteins. We investigated the distribution of adhesion components in cells grown on FN. All cultures were immunostained for actin (phalloidin), FAK, paxillin vinculin, p130Cas and c-src. No differences were observed in the localization of the focal adhesion components or signaling molecules (Fig. 2 and Supplemental Fig. 1). Interestingly, a relative decrease in the density of actin stress fibers was observed in PEA3(−/−) cells, suggesting a defect in actin dynamics (Fig. 2A–B). Supporting the actin phenotype, wildtype cells displayed more focal adhesion as assessed by immunostaining for FAK, paxillin and vinculin (Fig. 2C–H). Interestingly, little differences were observed for p130Cas and c-src localization (Supplemental Fig. 1). Although the overall number of adhesions was reduced in PEA3-null cells, their distribution was not affected. These results suggest that the observed motility defect is likely due to decreased focal adhesion signaling rather than mislocalization of adhesion components. The reduced number of adhesions may also account for the actin phenotype in PEA3-deficient cells.

![Fig. 2](Image of Figure 2)

**Fig. 2.** PEA3(−/−) cells display reduced numbers of focal adhesion complexes. WT and PEA3(−/−) cells were grown onto FN matrix, fixed and stained for actin stress fibers (A and B), FAK (C and D), paxillin (E and F) or vinculin (G and H). PEA3-null cells display a reduced density of stress fibers, focal adhesions but a normal distribution of focal adhesion complexes, as displayed by the focal adhesion marker staining (panels C–H). Similarly, no differences were observed for c-src and p130Cas (not shown). PEA3-reconstituted cell lines were indistinguishable from WT cells (not shown). The cells were photographed at 630×. (I) Focal adhesions were enumerated as per FAK staining. Adhesions were counted for at least 20 cells for both wildtype and PEA3-null cells (p<0.01).
components. Cell lysates from PEA3(−/−) and WT cells were surveyed for the expression of β1- and β3-integrin, vinculin, paxillin, PTEN, c-src, RhoA, Rac1, cdc42 and talin proteins (Fig. 3). Western blot analysis showed no major differences in the steady state levels of these different proteins. Furthermore, the levels of active Rac1, Cdc42 or Y31-phosphorylated paxillin were not altered (data not shown). Blotting for PEA3, confirmed the absence of PEA expression in the knock-out fibroblasts. Similar results were observed in a third PEA3-null line and a PEA3-reconstituted cell line (not shown). Combined with the observed reduction in focal adhesions, these results suggest that PEA3-null cells have an impaired ability to assemble focal adhesions rather than reduce levels in focal adhesion component expression. Because all 3 lines behaved similarly in motility assays and were indistinguishable in their adhesion marker expression profile, PEA3−/− cell line 1 was used in all subsequent experiments.

FN stimulation of fibroblasts has been shown to trigger the activation of FAK, the recruitment and activation of c-src and the formation of adhesion signaling complexes mediating actin rearrangements [22,23]. To test for potential adhesion signaling defects, FN-stimulated cells were analyzed for FAK activation. Total cell lysates from replated cells were immunoblotted for FAK phospho-Tyr397 (pY397), an autophosphorylation event indicative of FAK activation and focal adhesion turnover [14,17,24]. Western blot analysis (Fig. 4A) showed that FAK pY397 is transiently detected following replating on FN in WT cells (and in PEA3-reconstituted cells; not shown) as previously reported [23]. In contrast to WT cells, high levels of FAK pY397 were detected in all PEA3(−/−) cell lines throughout the replating time course, suggesting impaired turnover. In addition, high levels of FAK pY397 were also detectable in suspension cells, suggesting that PEA3-null cells fail to downregulate FAK signaling in suspension (Fig. 4A). Similarly, analysis of pFAK-Y925 levels shows that PEA3-null cells fail to re-induce tyrosine 925 phosphorylation 60 min following replating, supporting a turnover defect (Supplemental Fig. 2). We next investigated c-src activation following FN stimulation. Cell lysates were immunoblotted for c-src phospho-Tyr416 (c-src pY416), stripped and re-probed for total c-src. The two Tyr-416-positive species likely represent c-src and a cross reactive src-family kinase, possibly c-fyn (arrowheads). Reduced and delayed activation of c-src (pY416) was observed in PEA3(−/−) cells.

**Fig. 3.** Expression of focal adhesion components in PEA3(−/−) cells. Equal amounts of WT, PEA3(−/−)−1, and −2 lysates were resolved by SDS-PAGE, transferred onto PVDF membranes and immunoblotted for the indicated proteins. No significant changes in the protein expression levels of β1 and β3 integrin, vinculin, paxillin, PTEN, c-src and the Rho GTPases RhoA, Rac1, as well as talin were observed. Similarly, no differences were observed in PEA3-reconstituted cells and in a third PEA3-null isolates (not shown). PEA3 was not expressed in the PEA3−/− lines.

**Fig. 4.** Abnormal FAK and c-src regulation in PEA3(−/−) cells. (A) Cell lysates from time course replating assays on FN were surveyed for FAK phospho-Tyr397 (FAK pY397) and total FAK proteins. FAK pY397 is detected across the time course in PEA3(−/−) cells as well as in suspension (S). (B) Similarly, cell lysates were immunoblotted for c-src phospho-Tyr416 (c-src pY416), stripped and re-probed for total c-src. The two Tyr-416-positive species likely represent c-src and a cross reactive src-family kinase, possibly c-fyn (arrowheads). Reduced and delayed activation of c-src (pY416) was observed in PEA3(−/−) cells.

3.3. p130Cas tyrosine phosphorylation is impaired in the PEA3(−/−) cells

The scaffolding proteins p130Cas and paxillin have been shown to bind FAK, resulting in a FAK-src-mediated tyrosine phosphorylation [26–32]. As for FAK and c-src, paxillin is an important regulator of cell migration [33] and is necessary for efficient adhesion signaling [14,17,34]. Similarly, c-src-mediated phosphorylation of p130Cas has been shown to play an important role in the regulation of cell...
migration [27–29,35]. To investigate whether the migration deficit observed in PEA3-null cells affects adhesion complex formation and signaling, we examined the association of paxillin and p130Cas with the adhesion complex following FN stimulation. First, paxillin immunoprecipitates from stimulated cells were analyzed for p130Cas and c-src. Although no differences were observed in c-src association with paxillin containing complexes (Fig. 5A–C), PEA3-deficient cells weakly induced p130Cas tyrosine phosphorylation [29,35] upon FN stimulation (pp130Cas; Y249, Fig. 5B). Interestingly, we observed that more p130Cas could be co-immunoprecipitated with paxillin when PEA3−/− cells were replated onto poly-L-lysine (PL) matrix compared to WT and PEA3-reconstituted cells (Fig. 5B). We next immunoprecipitated FAK protein from FN stimulated lysates and immunoblotted for p130Cas. As for paxillin immunoprecipitates, western blot analysis showed that p130Cas remained associated with FAK for longer periods on FN but that c-src was unaffected (Fig. 6A–C; compare 20 and 30 min lanes). These results further support the notion that PEA3−/− cells exhibit focal adhesion signaling defects due to partial c-src activation. Overall, our data suggest that PEA3 target gene products may regulate c-src activity or focal adhesion assembly, thereby controlling signaling and migration.

4. Discussion

Previous studies have shown that PEA3-null tumors display poor metastatic potential, suggesting the existence of invasion or motility defects in these cells [7–9]. Here, we have shown that PEA3−/− cells fail to efficiently undergo wound closure and migrate up to 4-times less than WT cells or PEA3+/−-reconstituted cells in haptotaxis migration assays. Immunostaining of PEA3−/− cells grown on FN revealed a reduced number of focal adhesions but normal distribution of focal adhesion complexes, as visualized by FAK, paxillin and vinculin stains, suggesting that focal adhesion formation or assembly is impaired. Interestingly, a reduced density of actin stress fibers was observed in PEA3-null cells. It is likely that the reduced number of focal adhesions and aberrant focal adhesion signaling in these cells results in altered actin dynamics. We have also observed that PEA3−/− cells exhibit focal adhesion signaling defects characterized mainly by the failure to efficiently activate c-src, resulting in reduced p130Cas tyrosine phosphorylation and prolonged association with FAK. It is likely that low c-src activity contributes to the reduced levels of p130Cas phosphorylation at tyrosine 249 [35]. Furthermore, we showed that the impairment in focal adhesion signaling is not due to deregulation in c-src association with FAK or paxillin but rather c-src kinase activity.

C-src activation is accomplished in several ways including post-translational modification and molecular interactions [36]. Our results show that FAK and paxillin interactions with c-src are not altered in PEA3−/− cells, suggesting that c-src activation through such interactions does not contribute to the observed inactivation of c-src in PEA3−/− cells. One possibility is that kinases or phosphatases that directly or indirectly activate c-src are encoded by PEA3 target genes. In this study, only a subset of focal adhesion components was surveyed for cellular distribution. Therefore, there is still a possibility that the c-src-dependent recruitment of other adhesion proteins is impaired.

Several studies have shown that focal adhesion turnover is a microtubule-dependent process [37–40]. Microtubule depletion results in enlarged focal adhesions with high levels of FAK-pY397. In addition, microtubule regrowth induces cycles of FAK dephosphorylation and rephosphorylation on tyrosine 397, suggesting that this is associated with turnover [37]. Interestingly, both c-src and v-src were shown to be implicated in microtubule reorganization [41–43]. Therefore, one possibility is that PEA3-null cells do not efficiently recruit the microtubule network resulting in delayed focal adhesion turnover.
However, more extensive studies will have to be performed to address this possibility. Overall, our data suggest that the absence of pea3 gene expression does not alter the levels of a subset of adhesion markers, including FAK and the RhoGTPases. Interestingly, pea3 gene ablation may result in the downregulation of c-src regulators, leading to a decrease in FAK-src signaling, reduced levels of actin filaments, and an overall reduced migration.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2012.09.002.

List of abbreviations
FAK  focal adhesion kinase
FN  fibronectin
Y  tyrosine
Cas  Crk-associated substrate
MMTV  mouse mammary tumor virus

Acknowledgements
This work was supported by the Canadian Institute for Health Research, the Canadian Breast Cancer Research Alliance and MDAUSA. LAS is the recipient of a CIHR scholar award. ZC is supported by a CIHR studentship.

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