Integrins on eggs: focal adhesion kinase is activated at fertilization, forms a complex with integrins, and is necessary for cortex formation and cell cycle initiation

D. Chan, C. J. Thomas, V. J. Taylor, and R. D. Burke
Department of Biochemistry and Microbiology, University of Victoria, Victoria, BC V8P 5C2, Canada

ABSTRACT **We investigate the proposal that integrins and focal adhesion kinase (FAK) form a complex that has structural and signaling functions in eggs. FAK protein is present in eggs and is phosphorylated at fertilization. pY^{397}FAK localizes to the membrane 30 min after fertilization, which correlates with the expression of βC integrins and egg cortex development. The βC integrin and pY^{397}FAK coimmunoprecipitate from egg cortex lysates. PF573 228 and Y11, inhibitors of FAK, interfere with pronuclear fusion and reduce the abundance of pY^{397}FAK and cortical actin without affecting microvillar actin. Cyclin E normally accumulates in the nucleus 15 min after fertilization, then returns to background levels. PF573 228– or Y11-treated eggs accumulate cyclin E in the nucleus; however, levels remain high. In addition, PF573 228 interferes with the accumulation of pERK1/2 in the nucleus and in eggs initiating mitosis. Injection of eggs with a fusion protein consisting of the focal adhesion–targeting domain of FAK fused to green fluorescent protein interferes with cortex formation and produces abnormal nuclei. These data indicate that an integrin–FAK adhesion complex forms at the egg surface that functions in formation of actin arrays in the egg cortex and provides signaling inputs for cell cycle initiation.**

Monitoring Editor
William Bement
University of Wisconsin

Received: Mar 15, 2013
Revised: Jul 31, 2013
Accepted: Aug 20, 2013

INTRODUCTION

The eggs of a range of animals have been demonstrated to express integrins, and in numerous organisms mRNA encoding integrin subunits is stored as maternally derived mRNA (Lallier *et al.*, 1994). Expression of integrins on eggs before fertilization has led to thorough studies of a role in sperm–egg adhesion (Evans, 2009). In addition, there are indications that expression of integrins is necessary during early development (Brakebusch *et al.*, 1997). It is unclear, however, why eggs express integrins and what aspect of the function of these versatile and adaptable molecules is required during the specialized processes of early embryogenesis.

The egg cortex has several distinguishing properties that indicate that it is a specialized region of cytoplasm that includes the plasma membrane, an associated three-dimensional matrix of filamentous actin, and specialized elements of the endoplasmic reticulum (Sardet *et al.*, 2002). Although identified by early microscopists, the cortex only really became recognized when it was shown to retain many of its functions upon isolation (Mabuchi and Sakai, 1972; Vacquier, 1975; Schatten and Mazia, 1976). Dynamic changes in the mechanical properties of the cortex, such as stiffness and contractility, highlight a structural role (Chambers and Chambers, 1961; Sardet *et al.*, 1989; Mandato *et al.*, 2000). The cortex also has a role in localization of maternal mRNA and protein determinants, which emphasizes its critical role in development of the embryo (Elinson *et al.*, 1993; Weitzel *et al.*, 2004; Sardet *et al.*, 2005; Whalen *et al.*, 2012). The wide range of organisms that appear to have a

This article was published online ahead of print in MBoC in Press (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E13-03-0148) on August 28, 2013.

D.C., C.J.T., and V.J.T. developed concepts and approaches, performed experiments, analyzed data, and edited the manuscript. R.D.B. developed concepts and approaches, performed experiments, analyzed data, and prepared the manuscript.

Address correspondence to: Robert D. Burke (rburke@uvic.ca).

Abbreviations used: ATP, adenosine triphosphate; Cdk2, cyclin-dependent kinase 2; DAPI, 4′,6-diamidino-2-phenylindole; Dsh, dishevelled; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular regulated kinase; FAK, focal adhesion kinase; FATD, focal adhesion targeting domain; GFP, green fluorescent protein; MEK, mitogen-activated protein kinase kinase; pERK, phosphorylated ERK; pS^{19}MLC, phosphoserine 19 myosin light chain; pY^{397}FAK, phosphotyrosine 397 FAK.

© 2013 Chan *et al.* This article is distributed by The American Society for Cell Biology under license from the author(s). Two months after publication it is available to the public under an Attribution–Noncommercial–Share Alike 3.0 Unported Creative Commons License (http://creativecommons.org/licenses/by-nc-sa/3.0).
"ASCB®," "The American Society for Cell Biology®," and "Molecular Biology of the Cell®" are registered trademarks of The American Society of Cell Biology.

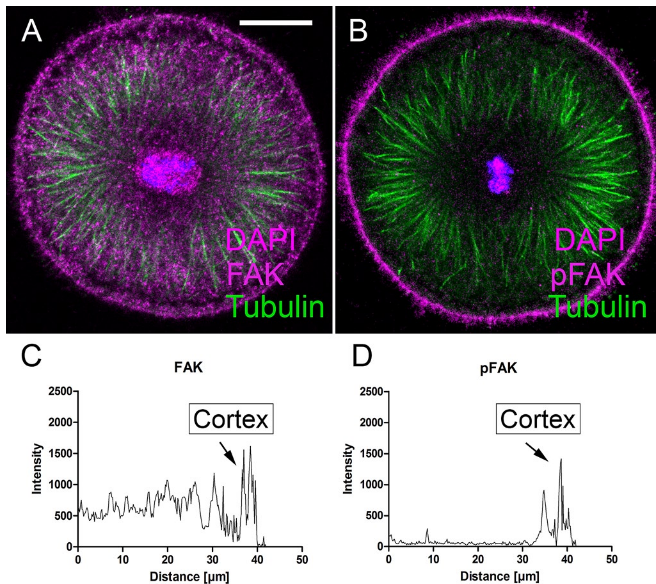


FIGURE 1: Eggs 90 min after fertilization reveal differences in the distribution of FAK and pY^{397}FAK. (A) Egg prepared with anti-FAK shows that immunoreactivity is granular and found throughout the cytoplasm. (B) Egg prepared with anti- pY^{397}FAK reveals that the phosphorylated form of FAK is restricted to the cell surface. (C, D) Intensity profiles along an arbitrary radius of the egg show differences in intensity throughout the cytoplasm and that most of the fluorescence at the membrane can be accounted for by pY^{397}FAK. Bar, 20 µm.

distinctive egg cortex suggests that it is a fundamental feature of animal development.

Urchin eggs were demonstrated to express integrins before fertilization, and they appear to be proteolytically removed as part of the cortical reaction during fertilization. Using antibodies to subunits, Murray *et al*. (2000) and Burke *et al*. (2004) documented aspects of the expression of integrins and demonstrated that integrin proteins are reexpressed within 30 min. Interfering with expression of the βC subunit reduced cortical arrays of actin, leading to the suggestion that integrins form a complex at the cell surface where they bind ligands in the hyaline layer and that the cortex of the sea urchin egg is anchored to a focal adhesion–like complex at the cell surface (Burke *et al*., 2004). Thus egg integrins and their associated adhesion complex may be critical components of the egg cortex.

Focal adhesion kinase (FAK) is expressed in early urchin development (Garcia *et al*., 2004). The demonstration by Garcia *et al*. (2004) that FAK is present in blastomeres at the apical surface prompted the hypothesis that FAK may interact with the βC integrins that are also expressed apically. Recently a role in cleavage has been postulated (Schumpert *et al*., 2013). Here we report that FAK is a component of this egg surface complex and is activated immediately after fertilization. We use small-molecule inhibitors and dominant-negative proteins to establish a requirement for FAK function in the elaboration of actin arrays in the cortex of the fertilized egg and show that FAK has a critical input into the pathways regulating reinitiation of the cell cycle.

RESULTS

FAK is activated at fertilization and redistributes to the egg cortex

Antibodies that recognize FAK without regard to its phosphorylation state indicate that eggs contain immunoreactive granules that are dispersed throughout the cytoplasm. In eggs fixed 90 min after fertilization, the cells are in early mitosis and FAK immunoreactive granules are in the cytoplasm and the cortex (Figure 1A). Cortical FAK is most clearly revealed as regions of higher fluorescence intensity in profile plots along a radius of the egg (Figure 1C). In contrast, in similar preparations with antibodies that recognize only FAK phosphorylated at a tyrosine residue in the activation loop (pY^{397}FAK; the activation loop is structurally conserved in sea urchin but is located between amino acids 450 and 475; Garcia *et al*., 2004), immunoreactivity is restricted to the egg cortex (Figure 1B). In preparations with anti-pY^{397}FAK, it is clear in images and radial intensity profiles that pY^{397}FAK is restricted to the egg plasmalemma and surface microvilli (Figure 1, B and D). In unfertilized eggs, pY^{397}FAK also localizes to thin, discontinuous patches of cortex (Figure 2). These data indicate that FAK protein is present in eggs and pY^{397}FAK is restricted to the egg cortex.

The egg cortex undergoes rearrangement immediately after fertilization and during the first cell cycle (Wong *et al*., 1997). We asked how the distribution of pY^{397}FAK correlates with changes in the distribution of filamentous actin and pMyosin light chain (pS^{19}MLC) between fertilization and 90 min. In unfertilized eggs, filamentous actin forms a thin, continuous layer associated with the membrane. In the first 30 min, the microvillar actin becomes more robust, and radially oriented actin filaments project 10–15 µm into the cortex (Figure 2). Radial microtubules are first apparent after 15 min and persist in the cortex until mitosis begins. The patches of pY^{397}FAK associated with the membrane before fertilization disappear by 5 min, and there is a marked increase in pY^{397}FAK in the cytoplasm throughout the first 30 min (Figure 2 and Supplemental Figure S1). Beginning at 15 min, pY^{397}FAK associates with the cortex, and by 30 min cortical pY^{397}FAK is prominent. Cortical pY^{397}FAK appears as 0.2- to 0.5-µm granules aggregated into clusters that form a layer 1 to 2 µm thick. Manders' overlap coefficient (0.819) and Pearson's *r* (0.695) indicate that actin and pY^{397}FAK colocalize in the cortex at this stage.

Immunoreactivity to anti-pS^{19}MLC first appears associated with the egg membrane 5 min after fertilization and increases in abundance throughout the first 60 min of development (Figure 2 and Supplemental Figure S1). Throughout the first cell cycle, pS^{19}MLC is restricted to the egg surface and microvilli. We conclude from these observations that the distributions of pY^{397}FAK, pS^{19}MLC, and actin change dynamically throughout the first cell cycle. In addition, the redistribution of pY^{397}FAK from the cytoplasm to the cortex of the egg correlates temporally and spatially with the reorganization of the actin cortex.

pY^{397}FAK associates with integrins in the cortex

It was previously demonstrated that βC-containing integrins are expressed within 30 min of fertilization and associate with the surface of the egg (Murray *et al*., 2000). Immunoprecipitates of detergent-solubilized egg cortices were used to determine whether pY^{397}FAK is physically associated with βC integrins. Using 1B10, a monoclonal antibody that binds the cytoplasmic domain of the βC subunit (Murray *et al*., 2000), in immunoprecipitations and subsequently probing with the same antibody, we identified a single band migrating at ~110 kDa in the egg cortex lysates and the bead fraction (Figure 3). When probed with anti-pY^{397}FAK, a band at ~125 kDa is revealed. In the reciprocal arrangement using anti-pY^{397}FAK, egg cortex lysates have an immunoreactive band at 125 kDa and similar bands in samples of material bound to beads in immunoprecipitates where either anti-pY^{397}FAK or anti-βC (1B10) was used as the primary antibody. Thus antibodies to βC integrins immunoprecipitate

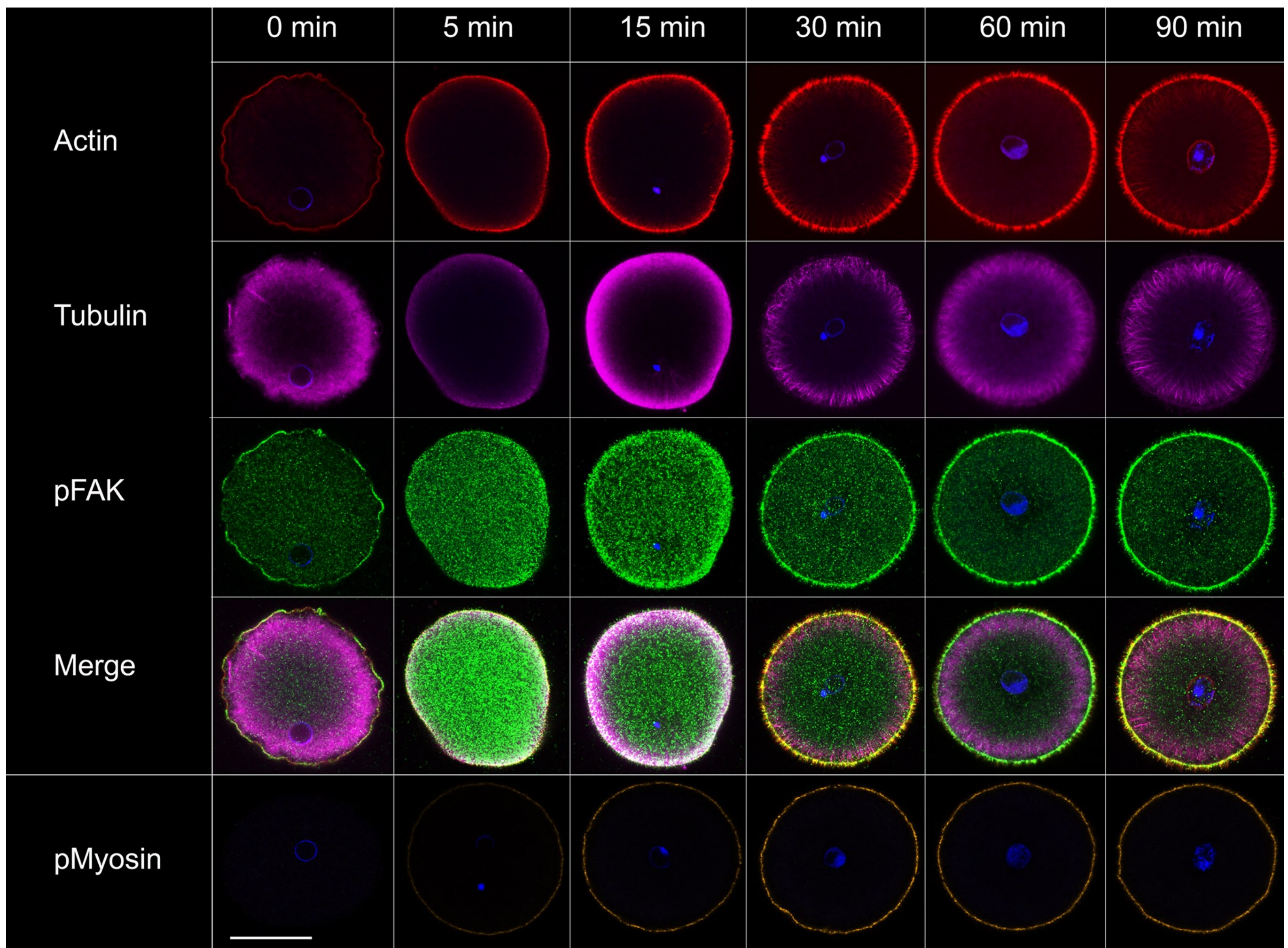


FIGURE 2: Confocal optical sections showing the chronology of cortex development. Eggs were fixed at intervals after fertilization (0 min is unfertilized), and samples of eggs were processed for immunofluorescence with phalloidin, anti-tubulin, and anti-pY^{397}FAK. A separate set was processed with anti–pS^{19} myosin light chain. Bar, 40 μm.

a complex containing pY^{397}FAK and antibodies to pY^{397}FAK immunoprecipitate a complex containing βC integrins. We conclude that pY^{397}FAK is physically associated with a complex containing βC integrins in the egg cortex.

FAK inhibitors interfere with pronuclear fusion and cortex development

PF573 228 is a small-molecule inhibitor of FAK that binds in the ATP-binding pocket and interferes with the catalytic activity of FAK (Slack-Davis *et al*., 2007). Unfertilized eggs treated with PF573 228 for 30 min before insemination appear to fertilize normally. Sperm adhere, the fertilization envelope and hyaline layers appear, and a single sperm nucleus enters the cytoplasm. The sperm head fails to swell to the same extent as in control preparations, however, and is often distant from the nucleus of the egg (Figure 4, A and B). By 60 min, the male pronucleus in PF573–228 treated eggs fails to fuse with the female pronucleus, and treated eggs rarely complete first cleavage. Y11 is a small-molecule inhibitor unrelated to PF573 228 that binds to the Y^{397} site of FAK and interferes with FAK function (Golubovskaya *et al*., 2012). Eggs treated with Y11 are delayed in fusion of the pronuclei, but by 45 min pronuclear fusion is completed; only a small percentage of eggs, however, will complete the first cleavage (Figure 4C). In experiments in which treatment with either Y11 or PF573 228 does not begin until 30 min after fertilization (Figure 4D), ~45% of eggs treated with Y11 complete first cleavage, but subsequent cleavages are abnormal and embryos fail to develop. PF573 228 blocks cleavage of eggs even when added after pronuclear fusion. We conclude from these experiments that inhibitors of FAK interfere with aspects of normal development during initiation of the first cell cycle.

We evaluated the reorganization of cortical actin in eggs treated with PF 573 228. In eggs fixed 90 min after fertilization, filamentous actin is in the microvilli that project 2–3 μm above the surface of the egg and in radially oriented arrays that project 5–6 μm into the cytoplasm (Figure 5). In eggs treated with PF573 228 or Y11, there is overall reduction in the abundance of cortical actin, as measured by fluorescent-phalloidin binding (Figure 5, A–C and J). Eggs treated with either inhibitor have microvillar actin that is similar to controls, but the cortical arrays of actin are significantly shorter in preparations of treated eggs (Figure 5, A–C and K). We also examined the effects of PF573 228 or Y11 treatment on the phosphorylation of FAK by comparing the levels of anti-pY^{397}FAK binding in treated and control preparations. In treated eggs, the distribution of pY^{397}FAK was identical to that in untreated eggs; however, there

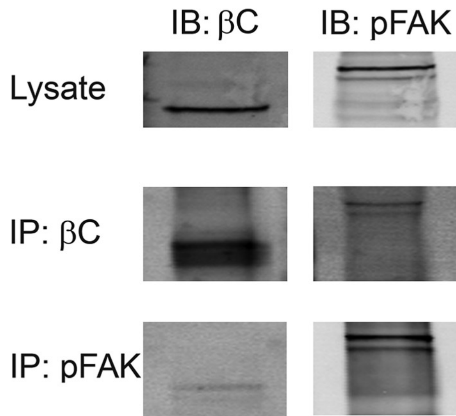
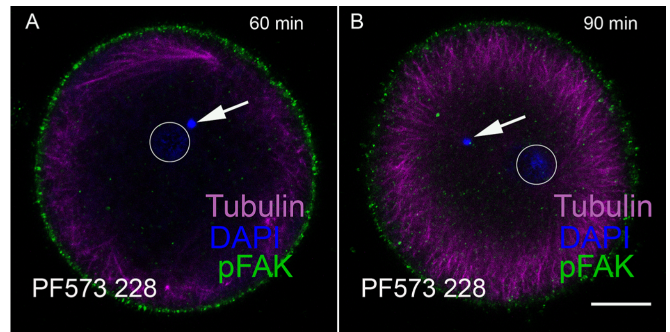
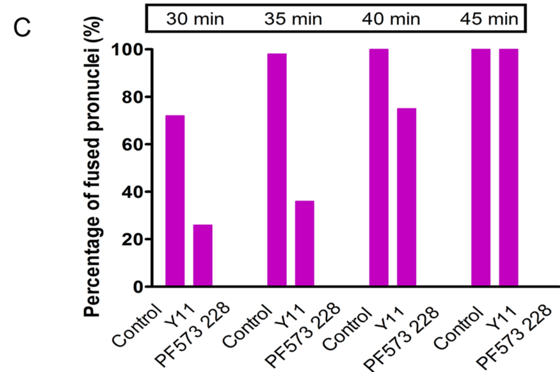


FIGURE 3: Immunoblots of 60-min egg cortex lysates and immunoprecipitates of egg cortex lysates showing a physical association of βC-containing integrins and pY^{397}FAK. Egg cortex lysates (top) contain immunoreactive bands at the predicted molecular weights (βC, 110 kDa; pY^{397}FAK, 125 kDa). Immunoprecipitates with anti-βC (middle) are enriched with immunoreactive material at110 kDa and contain an anti-pY^{397}FAK immunoreactive band. Similarly, blots of immunoprecipitates with anti-pY^{397}FAK (bottom) contain a band that is immunoreactive with anti-βC. All images are from a single experiment, run on a single gel, and double labeled with anti-βC and anti-pY^{397}FAK. Two levels of exposure were used for the pY^{397}FAK channel.





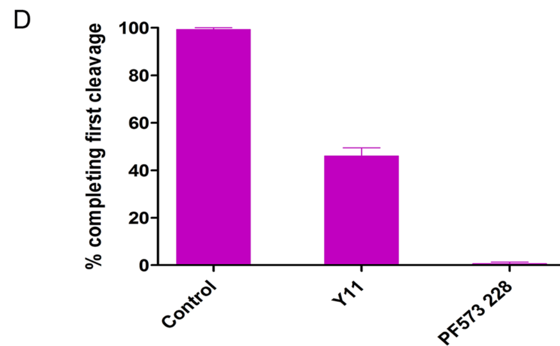


FIGURE 4: Fusion of pronuclei is affected by FAK inhibitors. (A, B) Confocal images of eggs prepared with DAPI, anti-tubulin, and anti- pY^{397}FAK to show a typical location of male (arrow) and female (circle) pronuclei at 60 and 90 min in eggs treated with the FAK inhibitor PF573 228. Bar, 20 µm. (C) Results of scoring pronuclear fusion in eggs (expressed as percentage) treated with either PF573 228 or Y11; time is minutes postfertilization. (D) To determine the effects of inhibitors subsequent to pronuclear fusion, inhibitors were added to eggs 30 min after fertilization. Although a zygotic nucleus formed, eggs did not cleave normally.

was a reduction in fluorescence intensity to 50–60% of control levels (Figure 5, D–F and L). The effect of PF573 228 on the abundance of pY^{397}FAK is also apparent from quantification of immunoblots (Supplemental Figure S2). Treatment with either inhibitor did not alter the distribution of pS^{19}MLC in eggs; however, there was an increase in the abundance of pS^{19}MLC when eggs were treated with either FAK inhibitor. Because integrin ligation is dependent on divalent cations, we rinsed fertilized eggs in calcium and magnesium–free seawater and after 90 min examined the abundance and distribution of pY^{397}FAK and actin (Figure 5, N–Q). Actin abundance is markedly reduced and restricted to the membrane, and pY^{397}FAK immunoreactivity is reduced to background levels by the lack of extracellular divalent cations, which implies that integrin function is necessary. Treatment with PF573 228 or Y11 did not have an effect on the abundance and distribution of βC integrins, atypical protein kinase C, or Dishevelled (Dsh; Supplemental Figure S3). We conclude from these experiments that FAK inhibitors alter the abundance and distribution of cortical actin, pY^{397}FAK, and pS^{19}MLC and these components of the cortex depend on extracellular divalent cations.

FAK inhibitors interfere with nuclear accumulation of cyclin E and pERK1/2

As part of the mechanism by which the cell cycle is reinitiated in urchin eggs, it was demonstrated that calcium-dependent activation of ERK1 promotes accumulation of cyclin E/cdk2 in male and female pronuclei and entry into S-phase of mitosis (Kisielewska *et al.*, 2009). To determine whether signaling from cortical integrin–FAK complex is critical to this process, we used an antibody to cyclin E (Sumerel *et al.*, 2001) to determine nuclear cyclin E abundance in the presence of a FAK inhibitor. Cyclin E immunoreactivity in the female pronucleus normally increases within the first 15 min after fertilization (Figure 6, A and B). By 35 min, these levels return to background and remain so throughout mitosis. In eggs treated with PF573 228, there is no increase in nuclear cyclin E immunoreactivity until 60 min after fertilization, and cyclin E immunoreactivity remains high until ~90 min after fertilization (Figure 6, A and B). When eggs are treated with Y11, cyclin E immunoreactivity increases through the first 60 min after fertilization and remains higher than in controls after 90 min (Figure 6B). Kisielewska *et al.* (2009) also demonstrated that nuclear accumulation of cyclin E is sensitive to the MEK inhibitor U0126 and roscovitine, a cdk2 inhibitor. With our antibody-based assay, these inhibitors block the increase in nuclear cyclin E immunoreactivity in a manner that is distinct from the effects we observe when eggs are treated with inhibitors for FAK (Figure 6C). We conclude from these experiments that inhibition of FAK interferes with the processes that regulate the nuclear accumulation of cyclin E.

The transient increase in cytoplasmic Ca^{2+} at fertilization is believed to activate ERK1, which accumulates in the nucleus 4–5 min

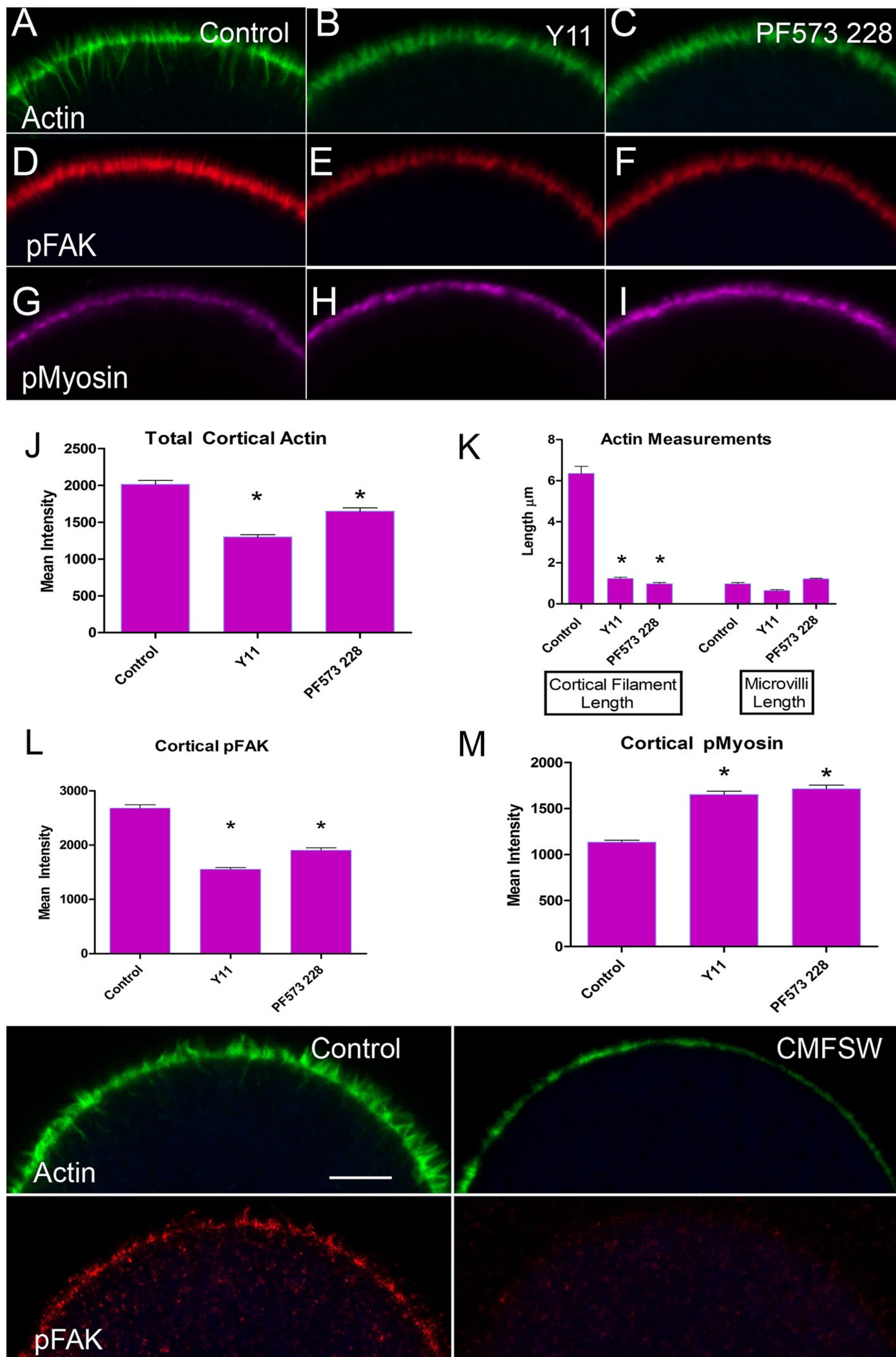


FIGURE 5: Inhibitors of FAK kinase activity interfere with the formation of cortical actin and accumulation of pY^{397}FAK (pFAK) but enhance accumulation of pS^{19}myosin light chain (pMyosin). (A–I) Representative confocal optical sections of eggs at 90 min postfertilization, untreated or treated with FAK inhibitorsY11 or PF573 228. (J–M) Quantification of images based on measurements of phalloidin fluorescence (J), linear measure of actin filament length (K), pFAK immunofluorescence (L), or pMyosin immunofluorescence (M). (N–Q) Confocal images of untreated eggs (Control) or eggs treated with Ca^{2+}, Mg^{2+}–free seawater (CMFSW) and prepared for immunofluorescence. The loss of pY^{397}FAK and reduction in actin abundance indicate that normal cortical development requires extracellular divalent cations, a common feature of integrin-mediated adhesion.

after fertilization (Philipova *et al.*, 2005; Zhang *et al.*, 2005). To determine whether inhibition of FAK activity is critical to ERK1 activation, we measured nuclear pERK through the first 30 min after fertilization. In untreated control eggs, we were able to measure brief accumulation of pERK1/2 in the nucleus 5 min after fertilization, which fades to background levels by 15 min after fertilization (Figure 7, A and B). In eggs treated with PF573 228, the levels of nuclear pERK are about half of those in the control eggs. With immunoblots in which whole egg lysates were probed with anti-pERK1/2, we find a similar pattern; PF573 228–treated eggs have less detectable pERK1/2. These experiments indicate that inhibition of FAK kinase activity with PF573 228 interferes with the activation of pERK.

Dominant-negative FAK protein interferes with cortex development

As an alternative approach to interfering with the function of FAK, we injected unfertilized eggs with a portion of FAK believed to act as a dominant negative (Hildebrand *et al.*, 1993; Ilić *et al.*, 1998) and then fertilized the eggs. When high levels of focal adhesion–targeting domain:GFP (FATD:GFP) fusion protein are injected, eggs failed to complete cleavage by 4 h (Figure 8, A and B). The eggs had numerous large aggregates of GFP-immunoreactive material dispersed throughout the cytoplasm. In injected eggs, there was no clear cortical actin and numerous regions of ectopic filamentous actin within the endoplasm. In several eggs, nuclei were fragmented or aberrantly shaped. In eggs injected with lower concentrations of FATD:GFP, GFP immunoreactivity was dispersed throughout the cytoplasm (Figure 8, C and D). The intensity of pY^{397}FAK immunoreactivity was reduced to ~50% of the levels of eggs injected with GFP alone, and there was discontinuous localization on the egg surface (Figure 8, C, D, and K). In addition, there was a reduction in the intensity of cortical fluorescent phalloidin compared with eggs injected with GFP alone (Figure 8, C, D, and K). 4′,6-Diamidino-2-phenylindole (DAPI) staining indicated aberrant nuclear shapes, and the arrangements of tubulin in mitotic asters was also abnormal in FATD:GFP–injected eggs (Figure 8, C and D). Eggs injected with full-length FAK:GFP protein completed cleavage and had GFP immunoreactivity dispersed throughout the cytoplasm; however, cortical GFP appeared to be enhanced (Figure 8, G and J). The measurements of pY^{397}FAK immunoreactivity and fluorescent phalloidin indicate lower levels than measured for GFP-injected eggs but levels higher than measured in FATD:GFP–injected eggs (Figure 8, E, H, and K). An additional control was to fertilize eggs and inject them with FATD:GFP after 60 min. In these preparations, the eggs underwent normal cleavage and had normal asters and what appeared to be normal levels of cortical actin and pY^{397}FAK

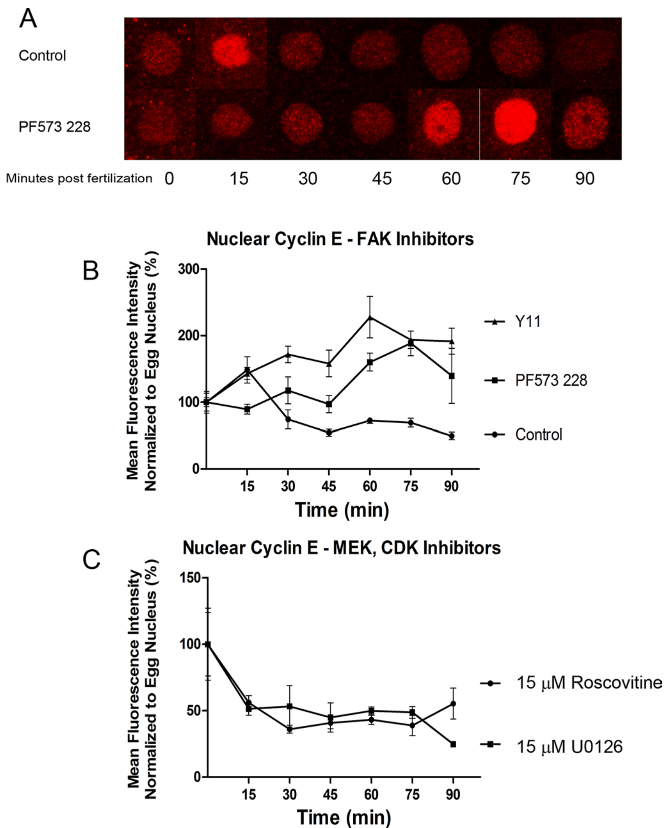


FIGURE 6: FAK inhibitors interfere with the normal pattern of accumulation of cyclin E in the nucleus of eggs. (A) Confocal optical sections of representative nuclei of eggs treated with FAK inhibitor PF573 228 and prepared for immunofluorescence with anti-cyclin E. (B) Quantification of cyclin E immunofluorescence expressed as a percentage of the mean fluorescence of unfertilized egg nuclei. Normally cyclin E accumulates in nuclei at ~15 min and returns to background levels as the nucleus enters S phase. Inhibitors of FAK cause a prolonged phase of accumulation. (C) Eggs were treated with inhibitors of MEK and cdk and quantified to provide a comparison.

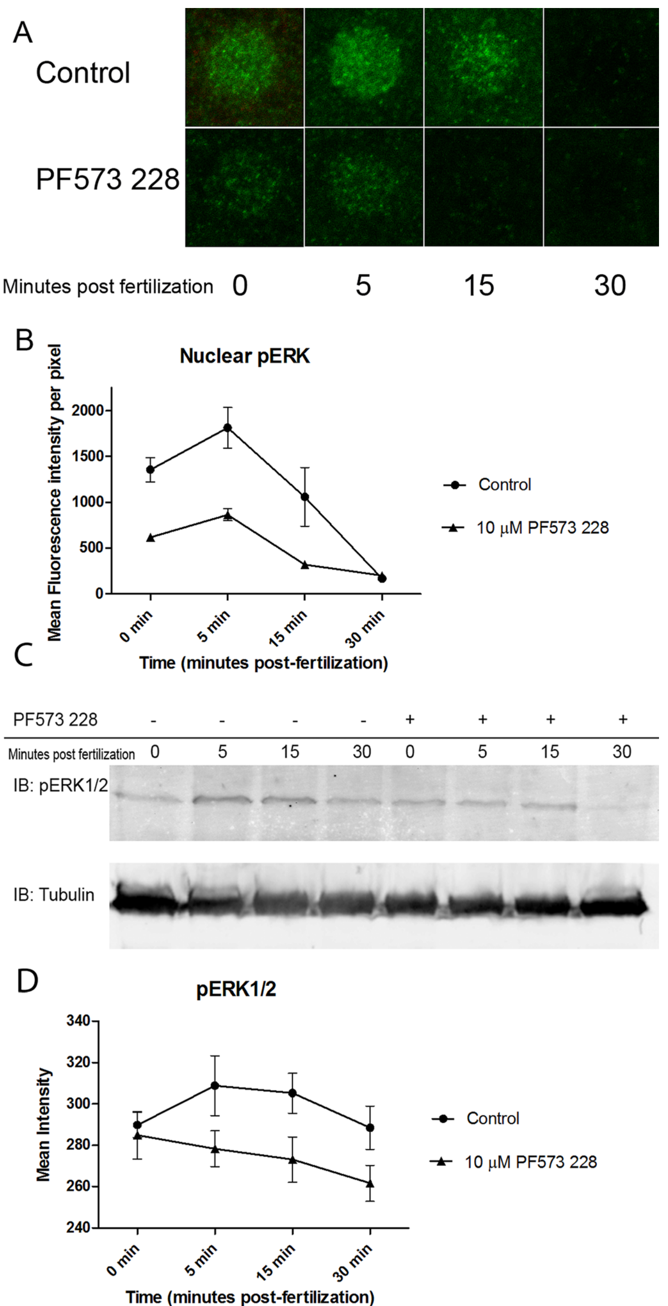


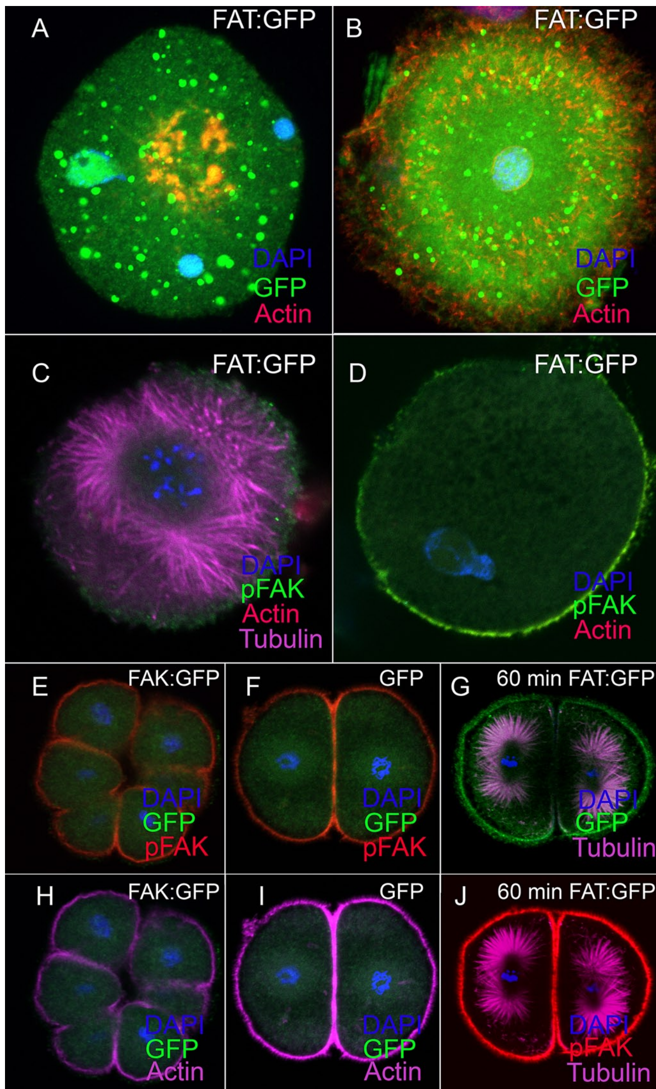
FIGURE 7: Treatment with the FAK inhibitor PF573 228 interferes with the accumulation of nuclear pERK in fertilized eggs. (A) Immunofluorescence preparations showing confocal optical sections of nuclei through the first 30 min after fertilization. (B) Quantification of preparations by the total fluorescence per pixel of treated and control nuclei. (C, D) Whole egg lysates of treated and control preparations as immunoblots that were quantified by measuring band density.

immunoreactivity. From these experiments we conclude that injection of FATD:GFP and FAK:GFP interferes with elaboration of the actin cortex and FATD:GFP appears to interfere with aspects of mitosis.

DISCUSSION

Cytoplasmic activation of FAK regulates pronuclei and cell cycle

Our data confirm that FAK protein is present in the egg (Garcia *et al*., 2004) and demonstrate that it is phosphorylated at Y^{397} while in the cytoplasm within the first 5 min of development. This immediate response to fertilization suggests that FAK may have a role in early developmental events. Treating eggs with FAK inhibitors during the interval that FAK is cytoplasmic interferes with pronuclear fusion and alters the pattern of nuclear accumulation of cyclin E. There are detailed studies of the mechanisms underlying the reinitiation of the cell cycle in urchin eggs (Philipova *et al*., 2005; Kisielewska *et al*., 2009). Kisielewska *et al*. (2009) demonstrated that Ca^{2+} activation of ERK1 promotes the coordinate accumulation of GFP-cyclin E and GFP-cdk2 in the egg pronucleus. In addition, their data indicate that cdk2 activity downstream of ERK1 activation is necessary for the initiation of S-phase and DNA synthesis. Philipova *et al*. (2005) note that inhibition of ERK1 prevents chromatin decondensation of the sperm chromatin after pronuclear fusion. Our data suggest that FAK may have input into this pathway (Figure 9). It is important to note, however, that the effect of blocking FAK with PF573 228 is distinctive, in that it blocks fusion of the male pronucleus, not only decondensation as reported by Philipova *et al*. (2005). It is also important to note that the FAK inhibitors do not block nuclear accumulation of cyclin E; they appear to interfere with the phasic nature of the increase. Treatment with either inhibitor causes an accumulation through the first 90 min of development. This pattern is distinctive from the pattern



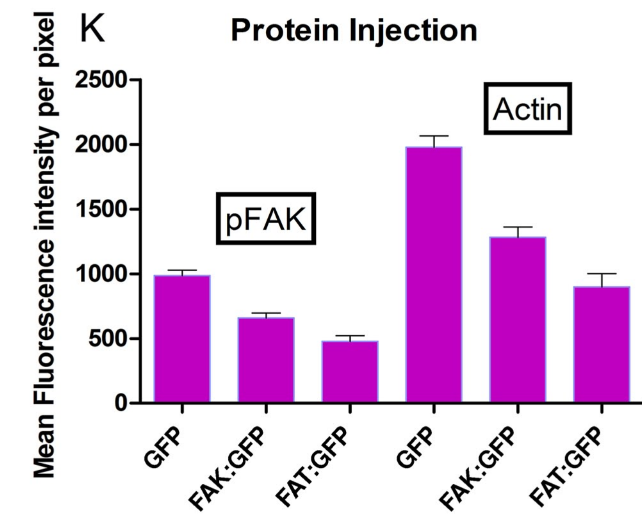


FIGURE 8: Injection of dominant-negative constructs of FAK and control proteins (FATD:GFP, FAK:GFP, GFP). (A, B) Unfertilized eggs injected with high concentrations of FATD:GFP and fixed 4 h postfertilization. Ectopic actin and aberrant nuclear morphology resulted. (C, D) Lower concentrations of protein were injected, and eggs had defects in formation of the cortex, membrane accumulation of pY^{397} FAK, and nuclear abnormalities. (E, H) Similar preparations of eggs injected with full-length FAK:GFP. (F, I) Preparations in which eggs were injected with GFP protein. (G, J) Preparations of eggs that developed for 60 min before injection with FATD:GFP protein, demonstrating that the dominant-negative effect is stage specific and appears to interfere with formation of the egg cortex. (K) Quantification of membrane-associated pFAK or membrane-associated actin in eggs injected with dominant-negative constructs.

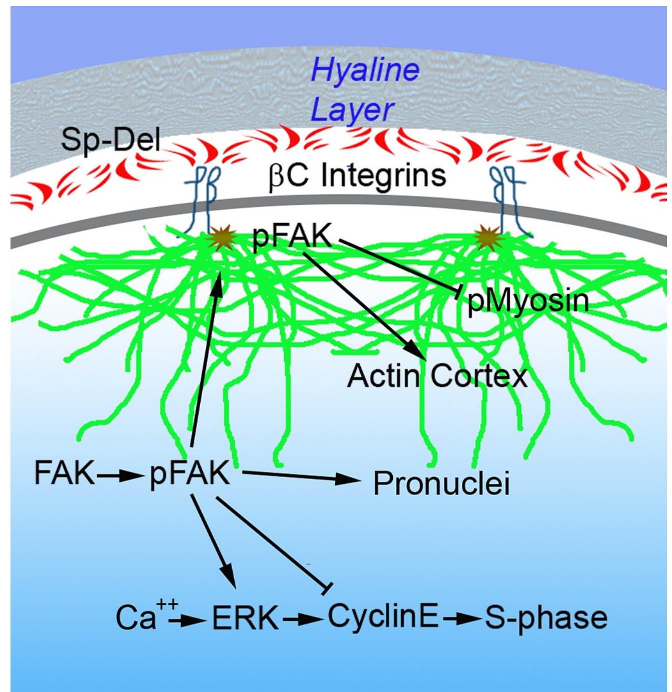


FIGURE 9: Summary of the roles of FAK after fertilization in sea urchin eggs. FAK is present in the cytoplasm and is phosphorylated immediately after fertilization. This activation appears to be necessary for the fusion of male and female pronuclei, and FAK has inputs into the MAP kinase regulation of the reinitiation of the cell cycle. Activation of FAK appears to be necessary for nuclearization of ERK and down-regulation of cyclin E. About 15 min after fertilization, βC integrins are expressed and bind Sp-Del in the extracellular hyaline layer. pFAK is recruited to an integrin-containing complex at the cell surface, where it appears necessary for the elaboration of the cytoplasmic arrays of cortical actin and regulation of phosphorylation of myosin.

of nuclear cyclin E seen with inhibitors of MEK or cdk. Thus our data indicate that during the first 15 min after fertilization, FAK has input into pronuclear fusion, enhances nuclearization of pERK, and is necessary for down-regulation of nuclear cyclin E (Figure 9).

Integrin and FAK are components of a cell surface adhesion complex

Within 15 min after fertilization, pY^{397}FAK begins to accumulate in the cortex and then at the egg surface. Previously it was demonstrated that βC-containing integrins appear on the membrane of the egg between 15 and 30 min after fertilization (Murray *et al.*, 2000; Burke *et al.*, 2004). Thus there is coordinate localization of integrins and pY^{397}FAK immediately after fertilization. The immunoprecipitation data establish that integrins and FAK are part of a molecular complex at the surface of the cell. Treating eggs with small-molecule inhibitors of FAK indicate a key role for FAK in formation of the egg

cortex. Treating eggs with either inhibitor reduces levels of pY^{397}FAK. Previous studies indicated that the inhibitors reduce the capacity of FAK for autophosphorylation at Y^{397} (Slack-Davis *et al.*, 2007; Golubovskaya *et al.*, 2012). Phosphorylation of FAK at Y^{397} is believed to be a necessary component of activation of kinase activity (Cox *et al.*, 2006). Our data indicate that after 15 min most of the active FAK is at the cell surface. Together these data indicate that within 30 min of fertilization, an adhesion complex containing βC integrins and pY^{397}FAK localizes to the surface of the egg.

Treating eggs with FAK inhibitors has an effect on the formation of the actin arrays in the egg cortex. The inhibitors appear not to have an effect on the actin cores of egg surface microvilli, yet they significantly reduce the amount and distribution of filamentous actin in the cortex. FAK is believed to have scaffolding functions in adhesion complexes, where the FATD domain is believed to interact with paxillin and talin (Chen *et al.*, 1995; Scheswohl *et al.*, 2008) and indirectly anchor actin (reviewed by Hall *et al.*, 2011). Thus we hypothesize that FAK has a similar function in eggs and that the FAK inhibitors interfere with the elaboration of actin arrays in the cortex by interfering with FAK's role in acting as a scaffold to anchor actin. A phosphorylated form of myosin (pS^{19}MLC) appears to be recruited to the egg membrane coordinately with integrins, pY^{397}FAK, and actin. Treatment with FAK inhibitors enhances either phosphorylation or recruitment of pS^{19}MLC to the cortex, suggesting that pY^{397}FAK has an inhibitory effect on pS^{19}MLC. Such a relationship is not unprecedented, as there are reports that indicate FAK and myosin are able to regulate each other in adhesion complexes (Pasapera *et al.*, 2010; Santos *et al.*, 2011). The small-molecule FAK inhibitors that we used indicate that FAK functions in the cortex of eggs in a conventional role; it is part of a membrane-associated complex that functions in elaboration of cortical actinomyosin arrays.

As an alternative approach to interfering with FAK function with small-molecule inhibitors, we used a dominant-negative form of FAK. Expression of the focal adhesion–targeting domain, a carboxy-terminal four-helix bundle, interferes with the function of endogenous FAK by competing for binding and disrupting scaffolding function (Ilić *et al.*, 1998). Our experiments indicate that FATD:GFP protein significantly reduces cortical actin and pY^{397}FAK and produces aberrant nuclear shapes. Full-length FAK:GFP produces a smaller but significant decrease in actin fluorescence in the cortex. Control preparations indicate that the effect is specific to injection of FATD:GFP or FAK:GFP and that the dominant-negative protein must be in the egg during the first 60 min of development to produce these effects. This is consistent with the dominant negative having an inhibitory effect during the formation of the cortex. Taken together, the coordinated appearance of cortex components and the loss of function, either with inhibitors or dominant-negative forms, is consistent with FAK having a critical structural role in formation of the egg cortex.

The cortex of somatic cells comprises a thin, actin-rich region beneath the membrane that is responsible for the mechanical properties of a cell (Salbreux *et al.*, 2012). Although an analogous structure, the egg cortex was recognized by early microscopists as a distinctive region of cytoplasm that extends up to 20 μm beneath the membrane (Sardet *et al.*, 2002). The cortex has dynamic mechanical properties, but, in addition, functions in the localization of egg determinants (Weitzel *et al.*, 2004; Sardet *et al.*, 2005; Tran *et al.*, 2012). Although FAK inhibitors appear to markedly reduce the actin cortex, there appears to be little effect on some protein components of the cortex. Par6 interacts with atypical protein kinase C (aPKC) and form a complex at the membrane associated with epithelial cell polarity (Alford *et al.*, 2009). Yet there was no apparent difference in the abundance or distribution of Par6 in eggs treated with PF573 228 (Supplemental Figure S3). Similarly, Dsh is a protein essential to axis specification in urchin eggs that is restricted to the vegetal cortex (Weitzel *et al.*, 2004). Interfering with the elaboration of the actin cortex appears not to affect the distribution of Dsh (Supplemental Figure S3). The dissociation of the pathways that operate in the egg cortex reveals the complexity and independence of the signaling that occurs there.

Egg surface adhesion complex functions like a focal adhesion

FAK is well established as a mediator of adhesion signaling (Hall *et al.*, 2011), but this is most commonly encountered in somatic cells at specialized adhesion complexes. We propose that a structure analogous to focal adhesions forms at the surface of the egg and serves as the interface between the extracellular matrix and the cytoskeleton (Figure 9). Integrin ligands have been identified in the extracellular layers of the fertilized egg (Alliegro and Alliegro, 2007; Zito *et al.*, 2010). Del-domain proteins are secreted from the egg within minutes of fertilization, producing the hyaline layer (Matese *et al.*, 1997). This layer is well established as an essential extracellular material to which cells adhere (Burke *et al.*, 1998; Wessel *et al.*, 1998). After 15 min, pY^{397}FAK localizes to the membrane at the same time integrins are expressed, ligands are secreted, and cortical arrays of actin form. pMyosin also appears to be recruited to this complex. In somatic cells, FAK is activated by integrin clustering, phosphorylated, and recruited to focal adhesions. In eggs, FAK is activated in the cytoplasm and appears to be recruited in a phosphorylated form to the cell surface complex. Signaling from focal adhesions in somatic cells is necessary for cell cycle progression (Damsky and Ilić, 2002). Adhesion complex signaling provides input to the mitogen-activated protein (MAP) kinase pathway that complements and is essential for cell cycle progression (reviewed by Lee and Juliano, 2004). The results of our loss-of-function experiments indicate a similar role for the egg surface integrin–FAK adhesion complex. Using two small-molecule inhibitors that target different aspects of FAK kinase function and injection of dominant-negative proteins, we consistently found interference with elaboration of the egg cortex and aspects of mitosis. Thus we propose that an adhesion complex forms at the surface of the sea urchin egg that is analogous structurally and functionally to the focal adhesion complexes of somatic cells.

There are several indications that egg surface coats and surface molecules are remarkably different (Vacquier, 1998). Integrins have been reported, however, on the surface of eggs of a range of species (DeSimone and Hynes, 1988; Gawantka *et al.*, 1992; He *et al.*, 2003). Functions associated with fertilization have been examined in detail (reviewed by Evans, 2009). Yet the presence of integrins in eggs as maternal message implies a function in early development. Some integrin subunits appear to be necessary for early development of mammalian eggs (Fassler and Meyer, 1995; Stephens *et al.*, 1995), but their role during cleavage stages is unclear. Given that the eggs of diverse species commonly have a cortex with often-robust arrays of actin (Sardet *et al.*, 2002), it is possible that the expression of an integrin–FAK complex in eggs, as demonstrated in urchins, is a common feature of metazoan eggs.

MATERIALS AND METHODS

PF573 228 and Y11 were from Tocris Bioscience (Ellisville, MO), and roscovitine and U0126 were from Sigma-Aldrich (St. Louis, MO). Anti–pSer^{19}-myosin light chain 2 was from Cell Signaling Technology (Beverly, MA). FAK (pY397) rabbit polyclonal antibody, Alexa

Fluor 488– and 568–phalloidin, Alexa Fluor 488 goat anti-mouse, and Alexa Fluor 568 goat anti-rabbit were from Invitrogen (Carlsbad, CA). Anti-pERK1/2 (pT202/pY204.22A), anti-Par6, and anti–α-tubulin were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to cyclin E were a gift from Bill Marzluff, University of North Carolina at Chapel Hill (Sumerel *et al.*, 2001). Murray *et al.* (2000) describe the anti-βC integrin antibodies 2D2 and 1B10. Anti-Dsh was generously provided by Athula Wikramanayake, University of Miami, Coral Gables. Anti-FAK was provided by Merrill Hille, University of Washington.

Adult *Strongylocentrotus purpuratus* were collected locally and kept in a recirculating seawater aquarium. Spawning was induced by intracoelomic injection of 0.55 M KCl or by shaking the urchin. Eggs were passed through a 150 μM Nitex filter, washed three times, and maintained at 13°C in filtered seawater. Sperm were collected dry and stored at 4°C.

For inhibitor experiments, eggs were incubated in 2.5 mM Y11 or 10 μM PF573 228 for 30 or 60 min before fertilization. Concentrations used were determined from published accounts (Slack-Davis *et al.*, 2007; Golubovskaya *et al.*, 2012) and by simple dose-response (cleavage after 2 h) experiments. We used the lowest concentrations of the inhibitors that consistently inhibited 50% of the eggs cleaving. The experiments with roscovitine and U0126 followed Kisielewska *et al.* (2009). If inhibitors required dimethyl sulfoxide (DMSO) to dissolve, the control preparation contained an equal concentration of DMSO. We added 2 mM 3-amino-1,2,4,-triazole (ATA) 15 min before adding a 1:10,000 dilution of sperm to the egg suspension to block fertilization envelope hardening. To monitor effects on cell cycle progression, we treated washed eggs for 15 min in ATA before fertilization and added inhibitors 30 min postfertilization. To determine the effects of inhibitors on pronuclear fusion or cleavage, we fixed treated and untreated eggs at intervals and prepared them with DAPI. For each experiment, 50–100 eggs at each time point were scored for pronuclear fusion, and experiments were replicated at least three times.

Immunofluorescence

Embryos were fixed for 10 min in PEM (Vielkind and Sweirenga, 1989; 4% paraformaldehyde in 100 mM 1,4-piperazinediethanesulfonic acid, 5 mM ethylene glycol tetraacetic acid, 2 mM $MgCl_2$, 0.2% Triton X-100, pH 6.8), washed with phosphate-buffered saline (PBS)–Tween (0.02% Tween 20, 1 mM Na_3VO_4, 1 mM NaF), resuspended in blocking buffer for 30 min (Superblock [Thermo Scientific, Waltham, MA] containing 0.03% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF), and incubated at 4°C overnight in primary antibody diluted in blocking buffer. Embryos were rinsed three times in PBS-Tween (15 min), incubated in secondary antibody diluted in blocking buffer for 60 min at room temperature, and rinsed three times in PBS-Tween. Specimens were mounted 1:1 in SlowFade Gold Antifade Reagent with DAPI (Invitrogen), and images were collected using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Jena, Germany).

Immunoprecipitates

Lysates of egg cortex (Walker *et al.*, 1994) were prepared in an immunoprecipitation buffer: 150 mM NaCl, 1 mM EDTA, 1% NP-40, and 25 mM Tris HCl, pH 7.4, containing 5 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Roche, Indianapolis, IN). Antibodies to integrins (1B10) or pY^{397}FAK (Invitrogen) were added to egg cortex lysates that had been precleared with immobilized protein A/G beads (Thermo Scientific Pierce, Rockford, IL). After 2-h or overnight incubation with antibodies, protein A/G beads were added, pelleted, and washed with immunoprecipitation buffer. After three washes, beads were resuspended in 1× PAGE sample buffer and the supernatant run on SDS–PAGE gels. An aliquot of lysate, immunoprecipitates with anti-integrin, anti- pY^{397}FAK, an irrelevant antibody, or no antibody was run on the same gel, and an immunoblot was prepared with either anti-βC (1B10) or anti-pY^{397}FAK. Images of the two channels of immunoblots were produced using Odyssey Imager software (LI-COR Biosciences, Lincoln, NE).

Dominant-negative constructs and protein expression

FATD:GFP, FAK:GFP, and GFP mRNA were prepared using an mMESSAGE mMACHINE SP6 Kit (Ambion, Austin, TX). Protein was expressed from RNA using an in vitro Protein Translation Kit (Thermo Scientific Pierce). Between 2 and 3 μg of mRNA was used as the initial template, and each 6-h reaction was spiked with 2 μg of mRNA at 2, 3, and 4 h. Fluorescence of expressed protein was confirmed and fluorescence quantified with ImageJ (National Institutes of Health, Bethesda, MD). Rhodamine B dextran was added to the protein solution before injection to confirm injections. Needles were filled with protein solutions so that 0.1–0.4 pg of the protein of interest was injected into each egg. Eggs were injected before fertilization or 60 min after fertilization and allowed to develop for up to 4 h, and embryos were prepared for immunofluorescence.

Image analysis

Quantification of fluorescence intensity was completed using ImageJ (MBF ImageJ 1.43m). Single, confocal optical sections were converted to grayscale before pixel intensity was measured. Mean pixel intensities were determined by selecting a small region of interest at five locations and recording the mean pixel intensity per unit area for at least 3 eggs/treatment. For measurements of fluorescence in the cortex and cytoplasm, a small square region of interest was used, and for measurements of nuclei, a circular area encompassing the entire nucleus was used. Microvillar and cytoplasmic actin length was determined using the line-drawing tool at 10 different locations per egg, and 4 eggs/treatment were analyzed. For pronuclear fusion and cell cycle progression experiments, 50 eggs/treatment were scored. Intensity data and measurements were analyzed and graphed using Prism 5 (GraphPad, La Jolla, CA).

ACKNOWLEDGMENTS

A Discovery Grant from the Natural Sciences and Engineering Research Council of Canada to R.D.B. supported this research. Athula Wikramanayake, Merrill Hille, and Bill Marzluff generously provided reagents.

REFERENCES

Alford LM, Ng MM, Burgess DR (2009). Cell polarity emerges at first cleavage in sea urchin embryos. Dev Biol 330, 12–20.

Alliegro MC, Alliegro MA (2007). Echinonectin is a Del-1-like molecule with regulated expression in sea urchin embryos. Gene Expr Patterns 7, 651–656.

Brakebusch C, Hirsch E, Potocnik A, Fassler R (1997). Genetic analysis of beta1 integrin function: confirmed, new and revised roles for a crucial family of cell adhesion molecules. J Cell Sci 110, 2895–2904.

Burke RD, Lail M, Nakajima Y (1998). The apical lamina and its role in cell adhesion in sea urchin embryos. Cell Adhes Commun 5, 97–108.

Burke RD, Murray G, Rise M, Wang D (2004). Integrins on eggs: the betaC subunit is essential for formation of the cortical actin cytoskeleton in sea urchin eggs. Dev Biol 265, 53–60.

Chambers R, Chambers EL (1961). Exploration into the Nature of the Living Cell, Cambridge, MA: Harvard University Press.

Chen HC, Appeddu PA, Parsons JT, Hildebrand JD, Schaller MD, Guan JL (1995). Interaction of focal adhesion kinase with cytoskeletal protein talin. J Biol Chem 270, 16995–16999.

Cox BD, Natarajan M, Stettner MR, Gladson CL (2006). New concepts regarding focal adhesion kinase promotion of cell migration and proliferation. J Cell Biochem 99, 35–52.
Damsky CH, Ilić D (2002). Integrin signaling: it's where the action is. Curr Opin Cell Biol 14, 594–602.
DeSimone DW, Hynes RO (1988). *Xenopus laevis* integrins. Structural conservation and evolutionary divergence of integrin beta subunits. J Biol Chem 263, 5333–5340.
Elinson RP, King ML, Forristall C (1993). Isolated vegetal cortex from *Xenopus* oocytes selectively retains localized mRNAs. Dev Biol 160, 554–562.
Evans JP (2009). Egg integrins: back in the game of mammalian fertilization. ACS Chem Biol 4, 321–323.
Fassler R, Meyer M (1995). Consequences of lack of beta 1 integrin gene expression in mice. Genes Dev 9, 1896–1908.
Garcia MG, Toney SJ, Hille MB (2004). Focal adhesion kinase (FAK) expression and phosphorylation in sea urchin embryos. Gene Expr Patterns 4, 223–234.
Gawantka V, Ellinger-Ziegelbauer H, Hausen P (1992). Beta 1-integrin is a maternal protein that is inserted into all newly formed plasma membranes during early *Xenopus* embryogenesis. Development 115, 595–605.
Golubovskaya VM *et al.* (2012). A small molecule focal adhesion kinase (FAK) inhibitor, targeting Y397 site: 1-(2-hydroxyethyl)-3,5,7-triaza-1-azoniatricyclo [3.3.1.1(3,7)]decane; bromide effectively inhibits FAK autophosphorylation activity and decreases cancer cell viability, clonogenicity and tumor growth in vivo. Carcinogenesis 33, 1004–1013.
Hall JE, Fu W, Schaller MD (2011). Focal adhesion kinase: exploring FAK structure to gain insight into function. Int Rev Cell Mol Biol 288, 185–225.
He ZY, Brakebusch C, Fassler R, Kreidberg JA, Primakoff P, Myles DG (2003). None of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm-egg binding and fusion. Dev Biol 254, 226–237.
Hildebrand JD, Schaller MD, Parsons JT (1993). Identification of sequences required for the efficient localization of the focal adhesion kinase, pp-125FAK, to cellular focal adhesions. J Cell Biol 123, 993–1005.
Ilić D, Almeida EA, Schlaepfer DD, Dazin P, Aizawa S, Damsky CH (1998). Extracellular matrix survival signals transduced by focal adhesion kinase suppress p53-mediated apoptosis. J Cell Biol 143, 547–560.
Kisielewska J, Philipova R, Huang JY, Whitaker M (2009). MAP kinase dependent cyclinE/cdk2 activity promotes DNA replication in early sea urchin embryos. Dev Biol 334, 383–394.
Lallier T, Hens MD, DeSimone DW (1994). Integrins in development. In: Integrins: Molecular and Biological Responses to the Extracellular Matrix, ed. DA Cheresh and RP Mecham, San Diego: Academic Press, 111–140.
Lee JW, Juliano R (2004). Mitogenic signal transduction by integrin- and growth factor receptor-mediated pathways. Mol Cells 17, 188–202.
Mabuchi I, Sakai H (1972). Cortex protein of sea-urchin eggs. 1. Its purification and other protein components of cortex. Dev Growth Differ 14, 247–261.
Mandato CA, Benink HA, Bement WM (2000). Microtubule-actomyosin interactions in cortical flow and cytokinesis. Cell Motil Cytoskeleton 45, 87–92.
Matese JC, Black S, McClay DR (1997). Regulated exocytosis and sequential construction of the extracellular matrix surrounding the sea urchin zygote. Dev Biol 186, 16–26.
Murray G, Reed C, Marsden M, Rise M, Wang D, Burke RD (2000). The alphaBbetaC integrin is expressed on the surface of the sea urchin egg and removed at fertilization. Dev Biol 227, 633–647.
Pasapera AM, Schneider IC, Rericha E, Schlaepfer DD, Waterman CM (2010). Myosin II activity regulates vinculin recruitment to focal adhesions through FAK-mediated paxillin phosphorylation. J Cell Biol 188, 877–890.
Philipova R, Kisielewska J, Lu P, Larman M, Huang JY, Whitaker M (2005). ERK1 activation is required for S-phase onset and cell cycle progression after fertilization in sea urchin embryos. Development 132, 579–589.
Salbreux G, Charras G, Paluch E (2012). Actin cortex mechanics and cellular morphogenesis. Trends Cell Biol 22, 536–545.
Santos AM *et al.* (2011). FERM domain interaction with myosin negatively regulates FAK in cardiomyocyte hypertrophy. Nat Chem Biol 8, 102–110.
Sardet C, Dru P, Prodon F (2005). Maternal determinants and mRNAs in the cortex of ascidian oocytes, zygotes and embryos. Biol Cell 97, 35–49.
Sardet C, Prodon F, Dumollard R, Chang P, Chenevert J (2002). Structure and function of the egg cortex from oogenesis through fertilization. Dev Biol 241, 1–23.
Sardet C, Speksnijder J, Inoue S, Jaffe L (1989). Fertilization and ooplasmic movements in the ascidian egg. Development 105, 237–249.
Schatten G, Mazia D (1976). The penetration of the spermatozoon through the sea urchin egg surface at fertilization. Observations from the outside on whole eggs and from the inside on isolated surfaces. Exp Cell Res 98, 325–337.
Scheswohl DM, Harrell JR, Rajfur Z, Gao G, Campbell SL, Schaller MD (2008). Multiple paxillin binding sites regulate FAK function. J Mol Signal 3, 1.
Schumpert B, Garcia MG, Wessel GM, Wordeman L, Hille MB (2013). Roles for focal adhesion kinase (FAK) in blastomere abscission and vesicle trafficking during cleavage in the sea urchin embryo. Mech Dev 130, 290–303.
Slack-Davis JK *et al.* (2007). Cellular characterization of a novel focal adhesion kinase inhibitor. J Biol Chem 282, 14845–14852.
Stephens LE, Sutherland AE, Klimanskaya IV, Andrieux A, Meneses J, Pedersen RA, Damsky CH (1995). Deletion of beta-1 integrins in mice results in inner cell mass failure and periimplantation lethality. Gene Dev 9, 1883–1895.
Sumerel JL, Moore JC, Schnackenberg BJ, Nichols JA, Canman JC, Wessel GM, Marzluff WF (2001). Cyclin E and its associated cdk activity do not cycle during early embryogenesis of the sea urchin. Dev Biol 234, 425–440.
Tran LD *et al.* (2012). Dynamic microtubules at the vegetal cortex predict the embryonic axis in zebrafish. Development 139, 3644–3652.
Vacquier VD (1975). The isolation of intact cortical granules from sea urchin eggs: calcium ions trigger granule discharge. Dev Biol 43, 62–74.
Vacquier VD (1998). Evolution of gamete recognition proteins. Science 281, 1995–1998.
Vielkind U, Swierenga SH (1989). A simple fixation procedure for immunofluorescent detection of different cytoskeletal components within the same cell. Histochemistry 91, 81–88.
Walker GR, Kane R, Burgess DR (1994). Isolation and characterization of a sea urchin zygote cortex that supports in vitro contraction and reactivation of furrowing. J Cell Sci 107, 2239–2248.
Weitzel HE, Illies MR, Byrum CA, Xu R, Wikramanayake AH, Ettensohn CA (2004). Differential stability of beta-catenin along the animal-vegetal axis of the sea urchin embryo mediated by Dishevelled. Development 131, 2947–2956.
Wessel GM, Berg L, Adelson DL, Cannon G, McClay DR (1998). A molecular analysis of hyalin–a substrate for cell adhesion in the hyaline layer of the sea urchin embryo. Dev Biol 193, 115–126.
Whalen K, Reitzel AM, Hamdoun A (2012). Actin polymerization controls the activation of multidrug efflux at fertilization by translocation and fine-scale positioning of ABCB1 on microvilli. Mol Biol Cell 23, 3663–3672.
Wong GK, Allen PG, Begg DA (1997). Dynamics of filamentous actin organization in the sea urchin egg cortex during early cleavage divisions: implications for the mechanism of cytokinesis. Cell Motil Cytoskeleton 36, 30–42.
Zhang WL, Huitorel P, Glass R, Fernandez-Serra M, Arnone MI, Chiri S, Picard A, Ciapa B (2005). A MAPK pathway is involved in the control of mitosis after fertilization of the sea urchin egg. Dev Biol 282, 192–206.
Zito F, Burke RD, Matranga V (2010). Pl-nectin, a discoidin family member, is a ligand for betaC integrins in the sea urchin embryo. Matrix Biol 29, 341–345.