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Atypical PKC, regulated by Rho GTPases and Mek/Erk, phosphorylates Ezrin during eight-cell embryo compaction

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ABSTRACT

Phosphorylation of Ezrin T567 plays an important role in eight-cell embryo compaction. Yet, it is not clear how Ezrin phosphorylation is regulated during embryo compaction. Here, we demonstrated that inhibition of Mek/Erk or protein kinase C (PKC) signaling reduced the phosphorylation level of Ezrin T567 in eight-cell compacted embryos. Interestingly, the Rho GTPase inhibitor C3-transferase caused basolateral enrichment of atypical PKC (aPKC), as well as basolateral shift of phosphorylated Ezrin, suggesting aPKC may be a key regulator of Ezrin phosphorylation. Moreover, inhibition of PKC, but not Mek/Erk or Rho GTPases, affected the maintenance of Ezrin phosphorylation in compacted embryos. We further identified that aPKC is indeed required for Ezrin phosphorylation in eight-cell embryos. Taken together, Rho GTPases facilitate the apical distribution of aPKC and Ezrin. Subsequently, aPKC and Mek/Erk work together to promote Ezrin phosphorylation at the apical region, which in turn mediates the apical enrichment of filamentous actin, stabilizing the polarized apical region and allowing embryo compaction. Our data also suggested that aPKC might be the Ezrin kinase during eight-cell embryo compaction.

Introduction

During mouse preimplantation embryogenesis, the first cell differentiation event leads to the segregation of the inner cell mass (ICM) and the trophectoderm (TE), which initiates after the compaction of blastomeres at the late eight-cell stage. Upon compaction, blastomeres become polarized (Ducibella and Anderson, 1975; Johnson and Ziomek, 1981; Lehtonen, 1980; Ziomek and Johnson, 1980). A polarized blastomere can give rise to two polarized outside cells through symmetric division, or one apolar inside cell and one polarized outside cell through asymmetric division. Subsequently, inside cells and outside cells develop into the ICM and the TE, respectively (Chen et al., 2010; Cockburn and Rossant, 2010; Johnson, 2009; Johnson and McConnell, 2004; Zernicka-Goetz et al., 2009). Cell polarization appears to be a critical factor for the cell fate choice. Down-regulation of polarity molecules, such as PAR3 and the atypical protein kinase C (aPKC), promotes inside localization of blastomeres and the ICM fate (Plusa et al., 2005).

Many proteins have been shown to participate in eight-cell blastomere polarization, including apically distributed JAM1, the polarity protein PAR3 and PAR6, aPKC, Erk2, Ezrin and filamentous actin (F-actin), as well as basolaterally localized PAR1 and E-cadherin (Louvet et al., 1996; Lu et al., 2008; Pauken and Capco, 2000; Thomas et al., 2004; Vestweber et al., 1987; Vinot et al., 2005). Ezrin is a member of the ERM (Ezrin, Radixin, Moesin) protein family, which serves as cross-linkers between F-actin and the plasma membrane (Chen et al., 1995; Kondo et al., 1997; Lamb et al., 1997; Martin et al., 1995; Takeuchi et al., 1994). The cross-linker function of Ezrin requires the phosphorylation of T567. In resting cells, Ezrin is in a dormant state, where the F-actin binding site is masked through the interaction between the N-terminal ERM homology (FERM) domain and the C-terminal tail domain. Phosphorylation of Ezrin T567 disrupts the interaction between the FERM and tail domains, exposing the F-actin binding site (Bretscher et al., 2002; Matsui et al., 1998; Shaw et al., 1998; Simons et al., 1998). Ezrin is expressed throughout preimplantation embryo development. It is distributed evenly around the cell cortex from the zygote to the early eight-cell stage, and becomes restricted to the apical region after compaction (Louvet et al., 1996). It has been suggested that blastomere

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polarization and compaction of eight-cell embryos rely on protein post-translational modifications, but not protein synthesis (Bloom and McConnell, 1990; Kidder and McLachlin, 1985; Levy et al., 1986). Consistently, phosphorylation of Ezrin T567 plays an important role in the compaction and the polarization of eight-cell embryos (Dard et al., 2004). The mutation of T567 into an aspartate (T567D), mimicking a phosphorylated residue, renders Ezrin to localize all around the cell cortex, instead of polarized apical distribution in eight-cell embryos. Consequently, the compaction of eight-cell embryos and the blastocoel formation in blastocysts are compromised by the Ezrin-T567D mutation. Oppositely, replacing T567 by an alanine (T567A), which prevents phosphorylation at this residue, does not affect compaction at the eight-cell stage. However, developmental defect is observed at the 16-cell stage and later, due to the aberrant redistribution of the Ezrin-T567A mutant to the basolateral cortex and reduced surface of adherens junctions (Dard et al., 2004).

Moreover, some signaling molecules, such as PKC, Mek/Erk and Rho family GTPases, are also involved in blastomere polarization and compaction of the eight-cell embryo (Clayton et al., 1999; Lu et al., 2008; Maekawa et al., 2007; Winkel et al., 1990). Both PKCζ and Erk2 show apical distribution in compacted eight-cell embryos (Lu et al., 2008; Pauken and Capco, 2000). Moreover, activation of PKC by phorbol ester leads to premature compaction at the four-cell stage, while inhibition of PKC with sphingosine blocks induced premature compaction as well as normal compaction of eight-cell embryos (Winkel et al., 1990). Mek/Erk signaling has been suggested to activate *Cdx2* and to suppress *Nanog*, thereby regulating the differentiation of the ICM and the TE (Lu et al., 2008). Yet, the functional significance of the apical distribution of Erk2 in late eight-cell embryos remains unclear. Is it the cause or the consequence of blastomere polarization? Although there is no evidence for asymmetric distribution of Rho GTPases in eight-cell embryos, it has been demonstrated that Rho GTPases are required for blastomere polarization and eight-cell embryo compaction (Clayton et al., 1999).

Even though many molecules involved in eight-cell blastomere polarization have been identified, the genetic and biochemical interactions of these molecules remain elusive. Here, we demonstrated that Mek/Erk, PKC and Rho GTPases regulate Ezrin T567 phosphorylation through different mechanisms. Rho GTPases regulate the distribution of aPKC, Ezrin and phosphorylated Ezrin (p-Ezrin). Both aPKC and Mek/Erk signaling are required for Ezrin T567 phosphorylation during embryo compaction. aPKC appears to be the Ezrin kinase in eight-cell embryos. Phosphorylation of Ezrin T567 in turn mediates the formation of F-actin at the apical surface, and promotes embryo compaction. Thus, our studies reveal a pivot role of Ezrin in eight-cell blastomere polarization and embryo compaction, as well as the regulation of Ezrin phosphorylation by multiple signaling pathways.

Materials and methods

Cell culture

V6.5 and iRasES Embryonic stem (ES) cells were cultured in Dulbecco's modified Eagle's medium (high glucose DMEM, GIBCO), supplemented with 15% fetal bovine serum (FBS, Hyclone), 2 mM L-glutamine, 5000 U/ml penicillin and streptomycin, 0.1 mM nonessential amino acids (Invitrogen), 0.1 mM 2-mercaptoethanol (Sigma), and 1000 U/ml LIF (ESGRO, Chemicon). To induce Ras expression, iRasES cells were plated on gelatin-coated tissue culture dish in mouse ES medium supplemented with 1 μg/ml doxycycline. ES-derived trophectoderm stem (ES–TS) cells (Lu et al., 2008) and trophectoderm stem (TS) cells were cultured in TS culturing medium containing RPMI 1640, 20% FBS (Hyclone), Fgf4 (25 ng/ml), heparin (1 ng/ml), 0.1 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM L-glutamine, penicillin and streptomycin (Invitrogen).

Phosphoproteomic analysis with mass spectrometry

Ras expression was induced in iRasES cells with 1 μg/ml doxycycline (Dox). Cells were harvested at indicated time points, and phosphoproteins were purified with Phosphoprotein Purification Kit (Qiagen). LC–MS/MS analysis was performed with the ThermoElectron Finnigan LTQ by the mass spec core facility in the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai.

Embryo culture

Female ICR mice (4–6 weeks) were induced to superovulate by intraperitoneal injections of 5 IU of pregnant mare serum gonadotropin (PMSG, Calbiochem) and 48 h later 5 IU human chorionic gonadotropin (hCG, Sigma). Then females were paired with ICR males overnight and checked for vaginal plugs the following morning. Two-cell embryos were flushed from oviducts at 42–48 h post-hCG and cultured in groups of 20–30 in a 50 μL droplet of potassium simplex optimization medium (KSOM) with amino acids (Millipore) covered by mineral oil (Sigma, for embryo culture) in a 37 °C incubator with 6.5% CO_2. Inhibitors were added at appropriate stages at the following concentrations: PD98059 (Calbiochem), 20 μM; PD0325901 (Sigma), 1 μM; C3-transferase (Cytoskeleton), 1 μg/ml; H-1152 (Calbiochem), 1 μM; cytochalasin D (Calbiochem), 0.5 μg/ml; D-Sphingosine (Sigma), 2.5 μM; Ro-31-8220 (Calbiochem), 5 μM; Gö 6976 (Calbiochem), 1 μM; PKCζ pseudosubstrate inhibitor (Myristoylated-SIYRRGARRWRKL-OH, Calbiochem), 10 μM. All experiments were performed with groups of more than 10 embryos and repeated three times.

Immunofluorescence

Embryos at desired stages were fixed in 4% paraformaldehyde for 20 min, and then permeabilized with 0.2% Triton X-100 for 30 min. After being blocked with 5% goat serum for 2 h, embryos were incubated with primary antibodies for 4–6 h at room temperature or overnight at 4 °C. Then embryos were washed and incubated with secondary antibodies and/or rhodamine-phalloidin (Molecular Probe). We used the following primary antibodies: anti-Ezrin (BD Transduction Laboratories), phospho-Ezrin (Thr567)/Radixin (Thr564)/Moesin (Thr558) antibody (Cell Signaling Technology), anti-E-Cadherin (BD Transduction Laboratories), anti-aPKC (Santa Cruz). We used Alexa Fluor 488 anti-mouse, Alexa Fluor 488 anti-rabbit and Alexa Fluor 594 anti-rabbit as secondary antibodies (Molecular Probe), and Hoechst 33342 (Sigma) for nuclei staining. Epifluorescent images were taken with Olympus IX81 microscope. Confocal images were captured using Leica TCS SP5 confocal microscope.

Western blot

Cells were lysed, and total protein concentration was measured using BCA Protein Assay Kit (Beyotime) to ensure equal loading in western blot analysis. The samples were resolved by SDS-PAGE followed by transferring onto a PVDF membrane (Millipore). Membranes were probed with anti-phospho-ERM antibody (Cell Signaling Technology), anti-Ezrin (Sigma), anti-Ras (Upstate), and anti-Actin (Abcam). Bound primary antibodies were recognized by HRP-linked secondary antibodies (GE Healthcare). HRP activity was detected by ECL Plus (Beyotime) and Kodak light film. For protein lysates from embryos, pools of