Plakoglobin is required for maintenance of the cortical actin skeleton in early *Xenopus* embryos and for cdc42-mediated wound healing

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Early *Xenopus* embryos are large, and during the egg to gastrula stages, when there is little extracellular matrix, the cytoskeletons of the individual blastomeres are thought to maintain their spherical architecture and provide scaffolding for the cellular movements of gastrulation. We showed previously that depletion of plakoglobin protein during the egg to gastrula stages caused collapse of embryonic architecture. Here, we show that this is due to loss of the cortical actin skeleton after depletion of plakoglobin, whereas the microtubule and cytokeratin skeletons are still present. As a functional assay for the actin skeleton, we show that wound healing, an actin-based behavior in embryos, is also abrogated by plakoglobin depletion. Both wound healing and the amount of cortical actin are enhanced by overexpression of plakoglobin. To begin to identify links between plakoglobin and the cortical actin polymerization machinery, we show here that the Rho family GTPase cdc42, is required for wound healing in the *Xenopus* blastula.

Myc-tagged cdc42 colocalizes with actin in purse-strings surrounding wounds. Overexpression of cdc42 dramatically enhances wound healing, whereas depletion of maternal cdc42 mRNA blocks it. In combinatorial experiments we show that cdc42 cannot rescue the effects of plakoglobin depletion, showing that plakoglobin is required for cdc42-mediated cortical actin assembly during wound healing. However, plakoglobin does rescue the effect of cdc42 depletion, suggesting that cdc42 somehow mediates the distribution or function of plakoglobin. Depletion of α-catenin does not remove the cortical actin skeleton, showing that plakoglobin does not mediate its effect by its known linkage through α-catenin to the actin skeleton. We conclude that in *Xenopus*, the actin skeleton is a major determinant of cell shape and overall architecture in the early embryo, and that plakoglobin plays an essential role in the assembly, maintenance, or organization of this cortical actin.

Introduction

Plakoglobin (also known as γ-catenin) is a member of the arm repeat family of proteins, and functions in both cell adhesion and signaling (for reviews see Ben-Ze’ev and Geiger, 1998; Hatzfeld, 1999). Plakoglobin is unique amongst the arm repeat family in that it is a component of both adherens junctions and desmosomes (Cowin et al., 1986). In adherens junctions, plakoglobin binds to the cytoplasmic COOH terminus of classical cadherins (Ozawa et al., 1989), whereas in desmosomes, it binds to desmosomal cadherins (Kowalczyk et al., 1997). Loss of function experiments have confirmed that plakoglobin plays roles in desmosome-based adhesion in the mouse (Bierkamp et al., 1996, 1999; Ruiz et al., 1996; Isac et al., 1999) and has multiple roles in early *Xenopus* embryos (Kofron et al., 1997). Overexpression experiments have also implicated plakoglobin in cell signaling (Karnovsky and Klymkowsky, 1995), a property of other arm repeat proteins, but this has not so far been confirmed in *Xenopus* by loss of function experiments (Kofron et al., 1997). Changes of plakoglobin levels have been observed in the progression of different kinds of carcinoma (Amitay et al., 2001; Pirinen et al., 2001), and plakoglobin can act as an oncogene (Kolligs et al., 2000). Plakoglobin has also been implicated in the autoimmune disease pemphigus (Caldelari et al., 2001).

In early *Xenopus* embryos, during the egg to blastula stage, all proteins are translated from mRNA, synthesized, and stored in the oocytes. In previous work, we depleted the mRNA-encoding plakoglobin from cultured *Xenopus* oocytes, which blocked the synthesis of plakoglobin protein in early embryos derived from these oocytes. This caused three marked effects. First, there was a modest effect on intercellular adhesion. Second, there was a dramatic defect in the general
cytoarchitecture of the embryo, which lost its spherical shape and its elasticity, and became flattened under its own weight. This effect seemed unlikely to be mediated through its binding to cadherins because EP-cadherin–depleted and α-catenin–depleted embryos did not show this effect, but did show dramatic loss of adhesion of inner cells of the embryo. Third, gastrulation was delayed. All these defects were rescued by injection of plakoglobin mRNA into the depleted oocytes or fertilized eggs. By the late gastrula stage, zygotic transcription of plakoglobin mRNA reached wild-type levels, and the embryos recovered and developed normally (Kofron et al., 1997).

In this paper, we further investigate the loss of architecture of the embryo. Little is known about the molecular basis of the cytoarchitecture of the large Xenopus embryonic cells, and so loss of blastula shape after plakoglobin depletion offered the opportunity to study this. Early Xenopus embryos, like those of other vertebrates, have little or no extracellular matrix until the gastrula stage, and so embryo architecture is likely to be controlled by the cytoskeleton of individual blastomeres. Of the three cytoskeletal filament systems, it is not known which system (or combination thereof) maintains the cytoarchitecture of the large blastomeres. Therefore, we analyzed the amounts and distributions of the major cytoskeletal elements in blastulae depleted of plakoglobin, and found that the microtubule and intermediate filament skeletons were not significantly different from control embryos, whereas the assembly of cortical actin in each blastomere was dramatically reduced. We also found that actin-dependent cell behavior in the embryo requires plakoglobin. Cortical actin has been shown previously to be an important component of embryonic and fetal wound healing (Bluemink, 1972; Martin and Lewis, 1992; Bement et al., 1993; Brock et al., 1996). Local assembly of cortical actin in the margins of wounds leads to their rapid closure by “purse-stringing.” We show here that plakoglobin is required for the formation of purse-strings, and for the repair of wounds in the blastula. Replacement of plakoglobin mRNA rescued wound healing and cortical actin assembly, whereas overexpression of plakoglobin caused increased cortical actin assembly and enhanced wound healing.

Cortical actin polymerization leading to cell motility is known to be controlled by the Rho family of GTPases (Hall, 1998; Bishop and Hall, 2000). In addition, cdc42 has been shown to induce actin assembly in Xenopus egg extracts, in a process that also requires N-WASP (Rohatgi et al., 1999). Therefore, we investigated the potential role of cdc42 in formation of the cortical actin skeleton and in wound healing. We found that cdc42 was required for the assembly of purse-strings and for normal wound healing in the Xenopus blastula. Myc-tagged Xenopus cdc42, expressed from an in-
jected mRNA, colocalized with newly polymerized actin in purse-strings, whereas overexpression of cdc42 enhanced wound healing, and depletion of the maternal mRNA blocked it. Overexpression of cdc42 did not rescue actin assembly and wound healing in plakoglobin-depleted blastula. However, plakoglobin overexpression did rescue the effect of cdc42 depletion. Taken together, this evidence shows that cdc42 is required to assemble an actin purse-string around the site of a wound, but cannot do so in the absence of plakoglobin, which is required for maintenance of the cortical actin skeleton of the blastula.

**Results**

In these experiments, we used an antisense oligo used previously to deplete plakoglobin mRNA in *Xenopus* oocytes, which in turn depletes plakoglobin protein from both soluble and membrane fractions of the embryo until the gastrula stage (Kofron et al., 1997). The degree of reduction of plakoglobin mRNA was identical to that found previously, and was assessed in this paper using real-time PCR (Fig. 1 A). Plakoglobin mRNA levels were quantified as described in Materials and methods, and normalized for the level of ornithine decarboxylase as a loading control. Plakoglobin mRNA

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**Figure 2. Microtubule and cytokeratin filament systems are still present after plakoglobin depletion.** (A–D) The microtubule (A and B) and cytokeratin (C and D) arrays in either uninjected (A and C), or plakoglobin depleted (B and D) blastulae. Microtubules are shown in the cells lining the roof of the blastocoel, whereas cytokeratin filaments are shown in the surface layer of animal cells. No significant differences were found after plakoglobin depletion.
was reduced to <10% of control levels using doses of 3–5 ng oligo per oocyte. This resulted in dramatic reduction in the level of plakoglobin protein, shown previously by Western blots (Kofron et al., 1997), and here by immunocytochemistry (Fig. 1, B and C). Fig. 1 (D and E) shows the effect on blastula architecture of these levels of depletion of maternally synthesized and stored plakoglobin mRNA. Embryos looked normal when inside their jelly coats and vitelline membranes, but when these were removed, the blastulae flattened against the substratum. The degree of flattening was shown by fixing devitellined embryos in 4% TCA, which hardens the embryos as well as fixes them, and photographing them from the side. The increase in diameter due to flattening ranged from 10 to 40% in 20 pairs of
Figure 4. **Wound healing and the cortical actin skeleton require plakoglobin.** Depletion of plakoglobin prevents healing of both animal caps (A), and the embryos from which they were removed (B). Top rows (A and B) are untreated, bottom rows are plakoglobin depleted. (B) Bottom row shows that injection of β-catenin mRNA does not rescue healing after plakoglobin depletion. Photographs were taken 1 h after dissection. (C) The rescue of wound healing in plakoglobin-depleted caps by the injection of plakoglobin mRNA and RLDX into one cell at the two-cell stage. The injected half of the animal cap (red) is healing normally, whereas the unrescued half (green) is not. (D–I) Actin patterns, stained using phalloidin, and recorded using the same settings on the confocal microscope, in control (D and E) and plakoglobin depleted (F and G), and rescued by injection of plakoglobin mRNA (H and I). Purse-strings around the wounds and cortical actin in individual blastomeres both require plakoglobin.
sibling-uninjected and plakoglobin-depleted embryos compared from several experiments.

Because blastulae contain little organized extracellular matrix material, it was most likely that the loss of blastula shape was caused by a cytoskeletal defect. Therefore, we stained isolated animal caps from fixed, plakoglobin-depleted blastulae using either anti-α-tubulin, or an antibody against the type II cytokeratin expressed early in *Xenopus* develop-

**Figure 5.** Overexpression of *plakoglobin* mRNA by injection of mRNA into both cells at the 2-cell stage causes an increase in cortical actin and more rapid wound healing. The animal cap from the uninjected embryo (A) has significantly less cortical actin than that from an embryo injected with 400 pg mRNA (B). (C) Bases from which the caps were removed. The plakoglobin-overexpressing bases heal faster than those of the uninjected controls. Picture was taken 15 min after excision of the caps. Notice that increasing doses of mRNA cause increasingly more spherical embryos.
Plakoglobin is required for the cortical actin skeleton and for wound healing

To study the actin skeleton, we stained isolated animal caps with fluorescently coupled phalloidin (Alexa 488–phalloidin). Fig. 3 shows the design of the experiment (Fig. 3, A–C) and the normal distribution of F-actin in the cortical cytoplasm of the animal cells lining the roof of the blastocoel at low (Fig. 3 D) and high (Fig. 3, E and F) magnifications. Actin filaments form a dense cortical network of intercrossing fibers in the cortex of each blastomere, in the plane of the surface membrane (Fig. 3 E). To identify deeper actin filaments, we stained animal caps cut through the animal pole, which allows better penetration of the stain into the cytoplasm of the blastomeres, and its visualization by the confocal microscope. Fig. 3 F shows three cells at the cut surface (one of which has an arrow), and shows that there is very little deep cytoplasmic filamentous actin. The great bulk of the actin skeleton is cortical and extends around the surface of each blastomere.

To provide a functional test of cortical actin polymerization, we investigated wound healing, because it is known to be actin-based in embryonic epithelia. Around the margin of a wound, an actin purse-string forms, and contracts to heal the wound (Brock et al., 1996). To find out if *Xenopus* blastulae heal wounds in this way, we excised animal caps from individual late blastula or early gastrula stage embryos. The circumference of the cap acts as a wound that heals rapidly by rounding up into a sphere, with the original external side remaining external (Fig. 4 A). The hole left in the rest of the embryo (referred to here as the base) also slowly heals by contraction of the circular edge of the wound (Fig. 4 B). Whole animal caps fixed between 5 and 15 min after excision, and stained with Alexa 488–phalloidin, revealed a dense accumulation of actin, a “circumferential purse-string,” surrounding the entire cap, beginning within 5 min of wounding (Fig. 3 D and Fig. 4, D and H).

Next, we examined the cortical actin skeleton, and wound-healing ability, of animal caps and bases from embryos whose maternal store of plakoglobin had been depleted, using doses of 3–5 ng oligo. Fig. 4 shows that both caps and the bases from which they were removed do not heal the circular wounds in the absence of maternally encoded plakoglobin. This effect can be rescued by reintroduction of plakoglobin mRNA. In the example shown in Fig. 4 C, 125 pg plakoglobin mRNA, mixed with the lineage tracer rhodamine lysine dextran (2 ng) was injected into the animal half of one cell of plakoglobin-depleted embryos at the two-cell stage. Animal caps were cut at the late blastula stage, fixed after being allowed to heal for 10 min, and examined under the confocal microscope. The injected sides of the animal caps healed rapidly, whereas the un.injected sides did not. Injection of mRNA encoding the closely related ARM-repeat β-catenin at levels that caused overdorsalization (400 pg) did not rescue wound healing (Fig. 4 B, bottom row of bases). Therefore, β-catenin and plakoglobin, although closely related, must have nonoverlapping functions with respect to polymerization of cortical actin. Fig. 4 (D–I) shows animal caps from control embryos (D and E), plakoglobin-depleted embryos (F and G), and plakoglobin-depleted embryos injected at the two-cell stage with 250 pg plakoglobin mRNA. Actin-rich purse-strings formed in control and plakoglobin-rescued caps, but did not form in caps from plakoglobin-depleted embryos (Fig. 4, D, F, and I, respectively). Pictures taken at higher magnification in the centers of the caps showed that the cortical actin skeletons of individual blastomeres were also substantially reduced in plakoglobin-depleted embryos and rescued by injection of plakoglobin mRNA (Fig. 4, E, G, and I). The comparative images shown in Fig. 4 (D–I) were taken using exactly the same settings on the confocal microscope.

The rescue by plakoglobin mRNA is an absolute test for specificity of this oligo-mediated effect because it formally excludes the possibility that the effect was caused by depleting a different mRNA or by nonspecific toxicity (neither of which would be rescued by plakoglobin mRNA). However, to further test the role of plakoglobin without the use of antisense oligos, we overexpressed plakoglobin mRNA by injection of either 200 or 400 pg into each cell at the two-cell stage. At the late blastula stage, the animal caps from these embryos were excised, fixed after 10 min, and stained with Alexa 488–phalloidin, whereas the bases were used to moni-
tor whether the rate of healing was altered. Fig. 5 shows that the plakoglobin-overexpressing bases healed more rapidly than control embryos, and the animal caps contained more polymerized cortical actin. Furthermore, the embryos showed greater rigidity and sphericalness than un.injected controls, in a dose-dependent manner (Fig. 5 C). Overexpression of plakoglobin therefore causes the reverse effect of its depletion.

Although cortical actin is significantly depleted in plakoglobin-depleted embryos, cell division still takes place, as is obvious from the fact that cells of the embryos shown above (Fig. 4 B) have the approximately normal numbers of cells. The fact that blastomers can still assemble an actin-rich contractile ring was shown by manually devitellining fertilized eggs just before the first cleavage division, fixing them as the first cleavage furrow formed, and then staining them with Alexa 488-phalloidin. Fig. 6 shows that contractile rings formed in plakoglobin-depleted embryos, even though sibling embryos, from this same batch of eggs, were unable to assemble purse-strings and heal wounds when they reached the late blastula stage, (the eggs shown in Fig. 6 are from the same batch of embryos shown in Fig. 4). We conclude that either sufficient cortical F-actin remains to assemble a contractile ring, or that this is assembled by a mechanism that does not require plakoglobin. G-actin is maintained at normal levels in plakoglobin-depleted embryos, as seen by Western blotting (unpublished data), so a lack of actin monomers is not the basis of the phenotype seen by plakoglobin depletion.

**Actin purse-string formation requires the GTPase cdc42**

Because cortical actin polymerization requires plakoglobin, we sought to identify whether it interacts with known components of the actin polymerization machinery. Purse-strings were found to form by a pathway including the small GTPase cdc42. To show this, we first constructed a full-length, myc-tagged *Xenopus* cdc42 cDNA, and injected the mRNA at doses between 20 and 125 pg, into either one cell, or both cells, at the two-cell stage, allowed the embryos to develop to the late blastula stage, and stained caps fixed either immediately, or after a healing time of 5 min after excision with anti-myc antibody and Alexa 488-phalloidin. Fig. 7 A shows that cdc42 becomes localized to the cortices of individual blastomeres derived from the injected cell. Fig. 7 B shows that the cdc42 also colocalizes with newly synthesized actin in the purse-string surrounding the cut cap. Next, we injected cdc42 mRNA into embryos at the two-cell stage, allowed them to develop to the late blastula stage, and excised animal caps. Embryos injected with cdc42 depleted caps faster than uninjected controls (Fig. 7, C and D). We designed antisense oligos against *Xenopus cdc42*, and depleted the maternal mRNA pool for this protein. Quantitative PCR (using a LightCycler®) showed that the oligo chosen (cdc42E) depleted the mRNA by 80% using 5 ng, and 90% using 10 ng, of unmodified oligo (unpublished data). Fig. 7 (E and F) show that both animal caps and bases from blastulae derived from oocytes injected with 8 ng cdc42E oligo healed more slowly than control embryos. Animal caps from control and cdc-depleted embryos were also fixed 5 min after excision, and stained with Alexa 488-phalloidin. Fig. 7 (G and H) show that no actin-rich purse-strings formed around the wounds in cdc42-depleted animal caps. These results show that overexpression of cdc42 results in more rapid wound healing, whereas its depletion has the opposite effect.

Finally, we performed a series of mRNA rescue experiments to show the relationship of cdc42 and plakoglobin functions. In this experiment, the bases were assayed for wound healing, whereas the caps were fixed 5 min after excision, and stained with Alexa 488 for cortical actin. Fig. 8 A shows that cdc42 mRNA rescued the effects of cdc42 depletion on wound healing. This demonstrated the specificity of this oligo-mediated effect. Fig. 8 B shows that injection of plakoglobin mRNA at the two-cell stage also rescued the effects of cdc42 depletion. Fig. 8 (C–G) shows that both cdc42 and plakoglobin depletion reduced cortical actin levels in animal cap cells, and that injection of either cdc42 or plakoglobin mRNAs restored cortical actin. In the reciprocal experiment, injection of cdc42 mRNA did not rescue the effect of plakoglobin depletion on wound healing (unpublished data).

**The cortical actin skeleton does not require linkage of α-catenin**

Plakoglobin acts as a link between cell surface cadherin and α-catenin, which in turn binds to the actin skeleton. This link is required for cadherin function (for review see Kemler, 1993). Depletion of the maternal mRNA-encoding α-catenin causes disaggregation of the blastomers, which would be expected from this known linkage, but does not cause loss of spherical architecture of the whole embryo (Kofron et al., 1997). Fig. 9 shows the effects of depleting α-catenin mRNA, using an oligo published previously (Kofron et al., 1997). The oligo significantly depleted the α-catenin protein as shown by Western blots (Fig. 9 C), and caused disaggregation of blastomers (Fig. 9 B). However, it did not cause loss of cytol structure (Fig. 9 A) nor loss of cortical actin (Fig. 9, D–F). This finding fitted well with our previous observation that α-catenin-depleted blastulae do not show collapse after removal of the vitelline membrane, even though the inner cells of the blastula are disaggregated (see Fig. 3 C in Kofron et al., 1997). We conclude that plakoglobin’s role in maintaining the cortical actin skeleton is not mediated through α-catenin.
Figure 8. The effect of depletion of *cdc42* mRNA on both wound-healing and cortical actin assembly in isolated animal caps, and the effects of injection of either *cdc42* or *plakoglobin* mRNA on the effects of *cdc42* depletion. (A) The effect of *cdc42* depletion on healing of bases (top row) compared to controls (middle row). Injection of 400 pg *cdc42* mRNA rescues the effect (bottom row). (B) Injection of 500 pg *plakoglobin* mRNA also rescues the effect (rows are the same as in A). (C–G) Pairs of images of isolated animal caps from the bases shown in A and B. On the left in each case are image stacks treated identically under the confocal microscope from Alexa 488–coupled phalloidin-stained caps, and on the right are quantitative measurements of pixel intensity across the image stack. (C) Uninjected; (D) *cdc42* depleted; (E) *plakoglobin* depleted; (F) *cdc42* depleted and injected at the two-cell stage with *cdc42* mRNA (400 pg); (G) *cdc42* depleted, and injected at the two-cell stage with *plakoglobin* mRNA (500 pg). Purse-strings and overall cortical actin depletion caused by depletion of *cdc42* are rescued both by *cdc42* and *plakoglobin* mRNAs.
Discussion

One of the principal findings in this work, and the others in the series (Heasman et al., 1994; Kofron et al., 1997), is that the three major catenins are required for very different functions at the egg to gastrula stages of the *Xenopus* embryo. The primary role of maternally encoded α-catenin is in intercellular adhesion, and the phenotype of its depletion is identical to that of EP-cadherin (Kofron et al., 1997). Maternally encoded β-catenin is primarily required for cell signaling in a wnt signaling pathway, and its depletion causes complete loss of dorsal structures, but does not affect cell adhesion or embryonic architecture (Heasman et al., 1994, Kofron et al., 1997). This does not exclude the possibility that β-catenin is also involved in cell adhesion because there is a stable store of maternal protein that remains membrane-bound until the blastula stage, when zygotic β-catenin begins to be expressed. In this work we show, by loss of function experiments, that the maternally encoded plakoglobin plays yet another role; that of maintenance of the cortical actin skeleton. Blastomeres are slightly less adherent than in control embryos, but the predominant effect is on embryonic shape. In these embryos, it is possible that β-catenin can take over the function of plakoglobin in cell adhesion. However, this cannot be tested at present by double depletion of the mRNAs due to the persistent membrane-bound store of β-catenin protein.

The second principal conclusion is that the cortical actin skeleton of the blastomeres plays the primary role in maintaining the overall architecture and rigidity of the early embryo. Although the blastula has dense networks of cytokeratin filaments (Klymkowsky et al., 1987; Torpey et al., 1992) and microtubules (Gard et al., 1995; Robb et al., 1996), these are not sufficient, in the absence of cortical actin, to maintain the overall cytoarchitecture of the embryo. This was shown indirectly by a previous observation. The cytokeratin filament network in the blastula is also encoded maternally, and its mRNA has been depleted (Torpey et al., 1992). Although the healing of superficial wounds was delayed, and the surface layer of cells failed to compact properly, there was no overall effect on embryo shape (see Fig. 3A in Torpey et al., 1992).

The third principal conclusion is that the cortical actin skeleton requires the presence of plakoglobin. How plakoglobin does this is unclear, and will be the subject of future work. Proteins known to bind plakoglobin to the surface membrane are the classical cadherins and desmosomal cadherins. Desmosomes have been identified between the most superficial cells of the embryo from the early blastula stage, where they form components of the junctional complexes that make the superficial cells of the blastula a functional epithelium (Sanders and Zalik, 1972; Perry, 1975; Muller and Hausen, 1995). However, they are not present in the deeper cells of the embryo (Angres et al., 1991; Gawantka et al., 1992). It is also known that cytokeratin networks in the superficial cells of the blastula are connected at cell boundaries by desmosomes (Muller and Hausen, 1995).
However, disruption of the cytokeratin networks themselves does not cause the architectural defect seen after plakoglobin depletion (Torpey et al., 1992), making it unlikely that the plakoglobin bound to these structures is playing this role.

There are many actin-bundling proteins known to play roles in assembly of cortical actin, including fimbrin, α-catenin, α-actinin, fascin, and the Rho family of small GTPases. Not all of these have been characterized in *Xenopus*, and so we cannot at this stage perform an exhaustive analysis of which may be involved in plakoglobin-mediated actin assembly. Instead, we have used the fragmentary data available in *Xenopus* to examine a selected few. The experiments reported here make it unlikely that plakoglobin maintains the actin skeleton through its binding to α-catenin because cortical actin is not removed by depletion of this mRNA, even in cells that become completely disaggregated from each other. We are currently testing the ability of a series of deletion plakoglobin constructs to rescue the effects of depletion, in an attempt to identify candidate binding partners. These can then be depleted to see if they mimic the effect of plakoglobin depletion. It has previously been shown that p120, a cadherin-binding, armadillo repeat protein related to β-catenin and plakoglobin, increases cortical actin-mediated activity in cells in culture (Grosheva et al., 2001), and that this function is independent of cadherin, but could be mediated through the Rho GTPase family (for review see Anastasiadis and Reynolds, 2001; Grosheva et al., 2001). Overexpression of p120 on the dorsal sides of early *Xenopus* embryos disrupts gastrulation movements (Paulson et al., 1997). Therefore, it is possible that plakoglobin either shares a function in cortical actin function with p120, or that the effect of depleting plakoglobin alters the function of p120. It will be interesting to see if the amount and distribution of p120 changes in plakoglobin-depleted embryos. Both β-catenin and plakoglobin have been shown to bind the actin-bundling protein fascin, independently of their interaction with cadherin (Tao et al., 1996). So it may be that plakoglobin is required by more than one element of the surface actin assembly machinery.

In the case of the Rho family GTPases, cdc42 had previously been shown to induce actin assembly in *Xenopus* egg extracts, in a process that also requires N-WASP (Rohatgi et al., 1999), and so we used this as a starting point for both gain and loss of function experiments. The results shown demonstrate conclusively that cdc42 plays a role in the actin polymerization in response to wounding. This is an interesting and novel finding, and it is now important to identify the other components in this pathway. The *Xenopus* blastula is an attractive model system for this because both gain and loss of function experiments can be performed. It will also be important to identify how the cdc42 pathway interacts with plakoglobin. The fact that overexpressed cdc42 does not rescue the effect of plakoglobin depletion shows that plakoglobin is required by the cdc42-containing pathway, but does not indicate how. The fact that plakoglobin overexpression does rescue wound-healing in cdc42-depleted embryos suggests that the role of cdc42 is to assemble plakoglobin or localize it in the right place for its function.

In conclusion, the loss of function experiments presented here show that plakoglobin and cdc42 are each required for wound healing, by playing essential roles in cortical actin polymerization. This is a novel and unexpected role for plakoglobin, unrelated to its action in cell adhesion, in establishing the cytoarchitecture of the early *Xenopus* embryo. This may be a specialization of the very large *Xenopus* embryonic cells, or may be of more general significance. Future experiments will be aimed at determining this, as well as identifying the domains of plakoglobin required for this function, and the proteins with which it associates.

### Table 1. PCR primers used

| PCR primer pair | Origin                  | Sequence                                                                 | Melt. temp | Annealing temp/time | Extension temp/time | Acquisition temp/time |
|-----------------|-------------------------|--------------------------------------------------------------------------|------------|---------------------|---------------------|-----------------------|
| ODC             | Heasman et al., 1994    | U: 5'-GCCATTGTGAAAGACTCCTCCATTCC-3'                                      | 95         | 55/5                | 72/12               | 83/3                  |
| cdc42           | Newly designed          | D: 5'-TTCCGCTGGATCTCCATTCC-3'                                            | 95         | 55/5                | 72/8                | 83/3                  |
| Plakoglobin     | Kofron et al., 1999     | U: 5'-GCTCGCCTGTAACACCACCATTCC-3'                                       | 95         | 60/10               | 72/16               | 85/3                  |
Materials and methods

**Xenopus oocyte and embryos**

Ovaries were surgically removed from *Xenopus laevis* females. Full-grown oocytes were manually removed from surrounding follicle cells and cultured in oocyte culture medium (240 ml Liebovitz medium, 160 ml distilled water, 0.16 g BSA, 2 ml glutamine [200 mM], and 200 μl gentamicin [10 mg/ml]). Antisense oligo was injected into the oocytes on day 1. Endogenous mRNA was maximally depleted, and the injected oligo was degraded, within 24 h of culture. Rescuing mRNAs were injected on day 2 of culture or into fertilized embryos at the two-cell stage. Injections were performed using a picoinjector (PL1-100; Medical Systems). Oocytes were matured with progesterone for 10–12 h and fertilized using the host transfer technique described previously (Holwill et al., 1987).

**Antisense oligonucleotides**

For plakoglobin, we used an 18-base, partially phosphorothioate-modified oligodeoxynucleotide described previously (Kofron et al., 1997). This was injected into oocytes at doses of 2–5 ng as described for each experiment. Plakoglobin: Oligo M7 5'-G*C*T*GCTGTGTGTATG*T*T*G-3' (asterisk represents phosphorothioate linkage). The phosphorothioate linkage in the first three and last three bases of the oligo was used to increase its stability in *Xenopus* oocytes. Oligos were diluted to 1 μg/μl with sterile distilled water and injected in doses from 0.6–5.0 ng per oocyte as described for each experiment.

For cdc42, five antisense oligos were tested for their ability to deplete maternal cdc42 mRNA. Oligo E (complementary to nucleotides 259–277 of the Xenopus cdc42 cDNA sequence in Genbank accession no. AF275252) was chosen for its high efficiency, purified by HPLC, and used to inject control cDNA was made and assayed in each LightCycler® run (Roche). Undiluted control cDNA and diluted control cDNA = 100%, 1:1 cDNA/H2O O = 50% and 1:10 cDNA/H2O O = 10%. In experiments where multiple embryonic stages were examined, the dilution series was used from cDNA of the uninjected control stage of development predicted to give the highest expression of the gene product being amplified. These values were entered as concentration standards in the LightCycler® sample input screen. Other controls included in each run were minus reverse transcriptase and water blanks. These were negative in all cases, but were not included in the figures for lack of space. LightCycler® quantification software (v3.0) was used to compare amplification in experimental samples during the log-linear phase to the standard curve from the dilution series of control cDNA. The comparisons are displayed as histograms. For each primer pair used, we optimized conditions so that melting curve analysis showed a single melting peak after amplification, indicating a specific product.

**Analysis of gene expression using real-time RT-PCR**

Total RNA was prepared from oocytes and embryos using the protease K method as described previously (Kofron et al., 1999). The primers used and cycling conditions are listed in Table I. To compare RNA levels of depleted and rescued embryos relative to controls, a dilution series of un.injected control cDNA was made and assayed in each LightCycler® run (Roche). Undiluted control cDNA = 100%, 1:1 cDNA/H2O O = 50% and 1:10 cDNA/H2O O = 10%. In experiments where multiple embryonic stages were examined, the dilution series was used from cDNA of the uninjected control stage of development predicted to give the highest expression of the gene product being amplified. These values were entered as concentration standards in the LightCycler® sample input screen. Other controls included in each run were minus reverse transcriptase and water blanks. These were negative in all cases, but were not included in the figures for lack of space. LightCycler® quantification software (v3.0) was used to compare amplification in experimental samples during the log-linear phase to the standard curve from the dilution series of control cDNA. The comparisons are displayed as histograms. For each primer pair used, we optimized conditions so that melting curve analysis showed a single melting peak after amplification, indicating a specific product.

**RNA**

Dr. Mike Klymkowsky provided *Xenopus* plakoglobin cDNA subcloned into vector SP64T. The construct was linearized with Smal and transcribed in vitro with T7-RNA polymerase. Plakoglobin synthetic RNA was diluted to a concentration of 1 μg/μl and injected in doses of 0.2–1.0 ng per oocyte.

**Wounding assays**

Late blastulae or early gastrulae were manually deevillified in 1X MMR and kept in 2% agar-coated petri dishes. Wounds were made in the animal cap with a sharpened tungsten needle.

**Immunostaining of animal cap explants**

Animal cap explants were dissected from devillified stage 9–10 embryos and fixed for 5 min in FG fix (3.7% formaldehyde/0.25% glutaraldehyde/2% Triton X-100 in Pipes buffer; Gard et al., 1997). Samples were immediately washed in two changes of 3-ml 1X PBST. Phalloidin staining was performed immediately on caps. Animal cap explants were incubated in 600 μl of 0.005 U Alexa 488-conjugated phalloidin/μl (Molecular Probes, Inc.) in PBST overnight at 4°C. Phalloidin-stained caps were washed for 1 h at RT in PBST and visualized by confocal microscopy (Table II). Samples for immunofluorescence were incubated for 1 h at RT with blocking buffer (10% horse serum, 4% BSA, and 0.5% fresh lysine), and stained under the conditions shown in Table II with different specific antibodies.

**Confocal microscopy**

Embryos, sections, or animal cap explants were observed on an Olympus BX40 equipped with a Bio-Rad MRC 1024 scanning confocal microscope, or a Zeiss LSM 510 on an Axivert 100M. Images shown are projections of Z-series at 1–3-μm intervals through 30 μm of the embryo or animal cap explant.

**Cloning of Xenopus cdc42 and synthesis of mRNA**

*Xenopus* cdc42 was cloned from an egg library via PCR amplification with primers designed to amplify the coding region. Two 3' primers were engineered. 5' WT synthesized the wild-type coding region, whereas 5' M encoded a single in-frame Myc epitope tag. The same 3' primer was used for both the wild-type and Myc epitope–containing clone. PCR amplification used the Advantage™ proofreading polymerase (CLONTECH Laboratories, Inc.). Fragments were subcloned into pCS2+ and sequenced to verify that there were no mutations resulting from the PCR amplification. Transcript was synthesized by linearizing with NotI and transcribing with the mMESSAGE mMACHINE™ SP6 kit (Ambion). Excellent technical help has been provided by Rachel Langland and Bertie Puck.

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| Antigen | Ab name | [1 Ab] | Time at RT | [2 Ab] | Time at RT |
|---------|---------|--------|------------|--------|------------|
| Plakoglobin | TD plako | 1:100 | 3 | FITC GAM 1:100 | 3 |
| Cytokeratin | CK7 | 1:50 | 3 | FITC GAR 1:100 | 3 |
| Tubulin | DM 1A | 1:250 | 3 | Cy5 GAM 1:500 | 3 |
| F-actin | OGP/A488P | 0.125 | 3 | NA | NA |

*Units: U/ml.*

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**Immunostaining of animal cap explants**

Animal cap explants were dissected from devillified stage 9–10 embryos and fixed for 5 min in FG fix (3.7% formaldehyde/0.25% glutaraldehyde/2% Triton X-100 in Pipes buffer; Gard et al., 1997). Samples were immediately washed in two changes of 3-ml 1X PBST. Phalloidin staining was performed immediately on caps. Animal cap explants were incubated in 600 μl of 0.005 U Alexa 488-conjugated phalloidin/μl (Molecular Probes, Inc.) in PBST overnight at 4°C. Phalloidin-stained caps were washed for 1 h at RT in PBST and visualized by confocal microscopy (Table II). Samples for immunofluorescence were incubated for 1 h at RT with blocking buffer (10% horse serum, 4% BSA, and 0.5% fresh lysine), and stained under the conditions shown in Table II with different specific antibodies.
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