Inca: a novel p21-activated kinase-associated protein required for cranial neural crest development

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Inca (induced in neural crest by AP2) is a novel protein discovered in a microarray screen for genes that are upregulated in Xenopus embryos by the transcriptional activator protein Tfap2a. It has no significant similarity to any known protein, but is conserved among vertebrates. In Xenopus, zebrafish and mouse embryos, Inca is expressed predominantly in the premigratory and migrating neural crest (NC). Knockdown experiments in frog and fish using antisense morpholinos reveal essential functions for Inca in a subset of NC cells that form craniofacial cartilage. Cells lacking Inca migrate successfully but fail to condense into skeletal primordia. Overexpression of Inca disrupts cortical actin and prevents formation of actin ‘purse strings’, which are required for wound healing in Xenopus embryos. We show that Inca physically interacts with p21-activated kinase 5 (PAK5), a known regulator of the actin cytoskeleton that is co-expressed with Inca in embryonic ectoderm, including in the NC. These results suggest that Inca and PAK5 cooperate in restructuring cytoskeletal organization and in the regulation of cell adhesion in the early embryo and in NC cells during craniofacial development.

KEY WORDS: Cartilage, PAK, Cortical actin, Cytoskeleton, Wound healing, Craniofacial, Tfap2a, Ectomesenchyme, Neural crest, Xenopus, Zebrafish

INTRODUCTION

Neural crest (NC) is a uniquely vertebrate cell type that arises at the boundary between neural plate and epidermis. During neural tube closure, NC cells delaminate from the neuroepithelium and begin to migrate as mesenchyme. This is accompanied by dramatic remodeling of cytoarchitecture and cell-cell adhesion, as NC cells migrate along predetermined pathways throughout the body (Duband et al., 1995). When NC cells finish migrating they differentiate into diverse derivatives including cartilage and bones of the skull, neurons and glia of the peripheral nervous system, melanocytes and many other cell types (Le Douarin and Kalcheim, 1999). Differentiation requires that many NC cells undergo second transformations in which they stop moving and condense, as in the formation of tightly packed cartilage elements or neuronal ganglia (Duband, 2006; Knight and Schilling, 2006; Richman and Lee, 2003). Although intensive research has focused on early NC induction, few studies have addressed the control of later NC development.

NC induction occurs through the combined effects of multiple signaling pathways, including bone morphogenetic proteins (BMP), Wnts, fibroblast growth factors and retinoic acid (Bastidas et al., 2004; LaBonne and Bronner-Fraser, 1998; Monsoro-Burq et al., 2005; Saint-Jeannet et al., 1997). These signals regulate expression of transcription factors that specify the NC phenotype (Huang and Saint-Jeannet, 2004; Meulemans and Bronner-Fraser, 2004; Sargent, 2006). Among these, Tfap2a, one member of a family encoding three to five closely related transcriptional activators (Eckert et al., 2005), plays an essential role in early specification of both epidermis and NC in Xenopus (Luo et al., 2003), and in numerous aspects of NC development in zebrafish (Barrallo-Gimeno et al., 2004; Knight et al., 2005; Knight et al., 2003) and mouse (Breuer et al., 2004). In Xenopus, Tfap2a can substitute for BMP signaling in NC induction (Luo et al., 2003). Several direct Tfap2a targets act in subsets of NC, such as Hoxa2 in skeletal progenitors (Maconochie et al., 1999) and C-kit (Kita) in pigment cell precursors (Huang et al., 1998; Wright et al., 2003), but other downstream NC effector genes are largely unknown.

We previously identified several novel targets of Tfap2a transactivation by microarray (Luo et al., 2005). One of these genes, Inca, is conserved among vertebrates, lacks any known structural domains (except for a 14-3-3 binding site of unknown significance) and has no known function. Inca is expressed in early cranial NC cells, and several other embryonic tissues including the presumptive epidermis, suggesting that it could be an important downstream effector of Tfap2a during embryogenesis.

Here we report that Inca is required for morphogenesis of NC-derived cartilage. Expression of Inca in cranial NC is conserved across multiple vertebrate species, including zebrafish, Xenopus and mouse. Knockdown of Inca expression by antisense morpholino oligonucleotides (MOs) in both Xenopus and zebrafish results in dramatic reductions in cranial NC-derived cartilage formation. We show that Inca interacts with PAK5, a p21-activated kinase downstream of the Rho GTPases Cdc42 and Rac1, previously implicated in cytoskeletal organization and apoptosis (Bokoch, 2003; Jaffer and Chernoff, 2002) as well as control of cell adhesion and convergent extension movements in Xenopus (Cau et al., 2001; Faure et al., 2005). Consistent with a role in cytoskeletal organization, Inca overexpression disrupts both cortical pigmentation in blastomeres and wound healing in Xenopus embryos. Combined overexpression of PAK5 and Inca enhances these phenotypes compared with either factor alone, suggesting that their association has functional significance for the control of cytoarchitecture. Taken together, our results suggest a novel mechanism in which Inca functions in concert with PAK5 to regulate the morphogenesis of cells in the early embryo, including postmigratory NC that form the craniofacial skeleton.

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Accepted 18 January 2007
MATERIALS AND METHODS

Embryo manipulation

Xenopus and zebrasfish embryos were obtained and maintained according to standard procedures (Sive, 1999; Westerfield, 1994). Injection and dexamethasone treatments of Xenopus animal caps at the midblastula stage with mRNA encoding chordin (Chd) (Sasai et al., 1994), Wnt3a (Wolda et al., 1993), a glucocorticoid-responsive Tfp2a fusion protein (GR-AP2) or dominant negative Tfp2a (dnTfp2a) (Luo et al., 2002) were performed as described previously (Luo et al., 2005). Blastula wound healing assays and rhodamine-conjugated phalloidin staining of animal caps were performed similarly to Kofron et al. (Kofron et al., 2002), with different healing times (Lloyd et al., 2005). Analysis of NC cells in zebrafish embryos was performed using a transgenic strain (sox10:eGFP) expressing enhanced green fluorescent protein (eGFP) in cranial NC precursors (Wada et al., 2003) and Dlx2 (Papalopulu and Kintner, 1993), and for zebrafish (Mizuseki et al., 1998), IncaA expression is strongly activated in NC cells during their migration into the dorsal neural tube (Fig. 2F,G) (Hausen and Riebesell, 1991). By expression begins at the one- and two-cell stage. Expression persists in NC cells during their migration into the dorsal neural tube. PH2). Similarly, in zebrafish, inca1 expression is zygotic and

Immunoprecipitation and western blotting

HEK293 cells and CHO cells were transiently transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). For immunoprecipitation, 24 hours after transfection, cells were lysed in lysis buffer [50 mM Tris pH 7.4, 150 mM NaCl, 1% Nonidet P-40 (NP40) with protease inhibitors (Roche)]. The lysates were incubated with a monoclonal antibody for a Myc-epitope (9E-10; Santa Cruz) for 2 hours, followed by protein-G Sepharose beads (Sigma) for another 2 hours at 4°C. The beads were washed three times in lysis buffer and analyzed by western blotting. To immunoprecipitate Inca protein from non-transfected Xenopus A6 cells, a rabbit antibody to the IncaA peptide DLPSTDVSPGCGQRQGLG conjugated to keyhole limpet hemocyanin was produced by Open Biosystems. Preimmune serum from the immunized rabbit was used as a negative control. Xenopus PAK5 antibody was a generous gift from N. Morin. Additional antibodies used were anti-phospho-Ser474-PAK4 (Cell Signaling Technology), anti-FLAG (Sigma) and anti-GFP (Sigma). Appropriate secondary antibodies were detected by enhanced chemiluminescence (Pierce).

RESULTS

Tfp2a regulates Inca in developing NC

Inca expression is strongly activated in Xenopus animal caps by a hormone-inducible Tfp2a (GR-AP2) (Luo et al., 2002). Animal caps isolated from embryos injected with a mixture of Chd (5 ng RNA per embryo), Wnt3a (250 pg per embryo) and GR-AP2 (600 pg RNA per embryo) differentiate as posterior neural plate if left alone, but form NC if dexamethasone is added during blastula stages (Luo et al., 2005). This upregulates expression of the NC marker Sox9 (Spokony et al., 2002) and reduces expression of the neural plate marker Sox2 (Mizuseki et al., 1998) (Fig. 1A). Inca expression is also strongly induced by dexamethasone. Note that there is a significant level of Inca expression in un.injected animal caps that form epidermis.

Inca cannot be assigned to a tentative gene family or function based on its protein sequence, but is the founding member of a group of Inca-related proteins conserved across vertebrates, including fish, frog, mouse and human. Sequence conservation averages 35-40% across species, and clusters near the proteins' center, including one highly conserved domain of 38 residues, the ‘inca-box’ (Fig. 1B). Incas also contain a highly conserved binding site for the scaffolding protein 14-3-3 (Muslin et al., 1996), located immediately upstream of the inca-box; however, preliminary evidence suggests that this site is not required for its activity in overexpression assays (see below; data not shown). Of two Inca-related genes in zebrafish, closely linked on chromosome 11, inca1 resembles Xenopus Inca slightly more than inca2 (Fig. 1C). In addition to one clear Inca ortholog in mouse (chromosome 9) and human (chromosome 3), another distantly related gene exists in fish (chromosome 8), frogs and mammals (mouse chromosome 3; human chromosome 1), with unknown function.

Fish, frog and mouse Inca orthologs have similar expression patterns during embryogenesis. Xenopus Inca expression begins shortly after midblastula transition (stage 9; Fig. 2A) and becomes localized to deep mesodermal cells and the inner, sensorial ectoderm layer during gastrulation (Fig. 2B). During neurulation, expression becomes restricted to notochord, epidermis and NC cells but is excluded from the neural plate or neural tube at later stages (Fig. 2C). Expression persists in NC cells during their migration into the pharyngeal arches (Fig. 2D-G), where it is confined to NC-derived head mesenchyme (Fig. 2F,G) (Hausen and Riebesell, 1991). By early tadpole stages, Inca is expressed in cranial ganglia and in the dorsal eye vesicle in addition to pharyngeal arch mesenchyme (Fig. 2H). Similarly, in zebrafish, inca1 expression is zygotic and

In situ hybridization, Alcian Blue staining and TUNEL staining

Whole-mount in situ hybridization was performed as described previously in Xenopus (Harland, 1991), zebrafish (Thissle et al., 1993) and mouse (Sage et al., 1996). Digoxigenin-labeled antisense probes used for Xenopus were IncaA, Tfp2a, Tfp2b, Wnt3a, Sox2, Sox9, Sox10, Sox11, Sox14, Sox17, Sox19, Sox20, Sox21, Sox22 and Dlx2 (Papalopulu and Kintner, 1993), and for zebrasfish inca1 (GenBank Accession Number, NC018555). Embryo sections in JB4 followed manufacturer instructions (Polysciences). Alcian blue cartilage staining was performed as described previously (Pasqualetti et al., 2000). The TUNEL assay was performed on whole-mount embryos as described by Hensey and Gautier, 1998, except that epidermis was manually removed from tadpoles after fixation to facilitate reagent penetration.

Interaction of Xenopus Inca and PAK5 in yeast

The mouse Inca ORF was used as bait in a two-hybrid screen with a mouse E11-stage cDNA library according to manufacturer instructions (Matchmaker™ Two-Hybrid System; Clontech). Xenopus Inca and PAK5 ORFs were also cloned into pGBK7 and pGAD7, respectively. Yeast transformation was performed using standard procedures.

DNA constructs and RNA synthesis for injection

Two Xenopus Inca pseudoalleles (IncaA and IncaB) and zebrasfish inca1 and inca2 were identified by BLAST searches and full-length expressed sequence tag (EST) clones were obtained (Open Biosystems). GenBank Accession Numbers are given in the legend to Fig. 1. Open reading frames (ORF) of IncaA and IncaB were sub-cloned into the EcoRI-XhoI sites of pCIS (Dehedy et al., 1999). Full-length sense mRNAs were generated by plasmid digestion with NotI and transcription by SP6 polymerase (Ambion Message Machine). IncaA-GFP and IncaB-GFP were generated by PCR and used to create in-frame N-terminal fusions to pCS2+eGFP and pDSRed1-N1 (Clontech), respectively. Xenopus PAK5-GFP and related constructs have been previously described (Cau et al., 2001).

Antisense MOs

 Translation-blocking antisense MOs were used for both Xenopus and zebrasfish embryos (GeneTools). MO sequences (start codon underlined) for Xenopus were: GCCGATCCACCAAGCGAGGAGAT (IncaA MO), ATGCCATTTGTGATCACCCAGG (IncaB MO), CAGACACGCAGTAAGTTGCCCCG (IncaA MO2) and AGATGAGACTGCGGAGAATGCTCCC (IncaB MO2). A MO containing five mismatched base pairs from the IncaA MO was used as a control (GCTCATACGGAACGGGACTAC; mismatched nucleotides in lowercase). MOs were injected into one or two blastomeres at the two-cell stage, along with 200 pg luc2 mRNA as a lineage tracer when a single blastomere was injected. The total in all cases was 10 ng of each MO. To assay MO effectiveness in Xenopus, plasmids containing the MO binding site and N-terminus of IncaA (base pairs –30 to +948) and IncaB (base pairs –21 to +402) fused to the N-terminus of GFP were co-injected with MOs into one- and two-cell stage Xenopus embryos. Zebrafish embryos were injected with 10 ng of MO (5′-TGCGAGACACACATTTCATTAAT-3′) at the one- and two-cell stage.

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Inca required for craniofacial development

Inca is a novel Tfp2a target in the NC. (A) Animal caps were injected with Wnt3a, Chd and GR-AP2a mRNA, treated with 10 μM dexamethasone (DEX+) or solvent alone (DEX−), and subjected to northern blot analysis using probes as indicated, with 18S rRNA as a loading control. Ul, uninjected. (B) Predicted protein sequence alignment for Xenopus (X), zebrafish (Z), mouse (M) and human (H) Inca. Identical residues are shaded. The conserved 38-residue inca-box is underlined, and the 14-3-3 binding site is indicated with stars. (C) Dendrogram generated using ClustalW (http://clustalw.genome.jp). Accession Numbers are: Xenopus IncaA, BI092564. Mouse, AK076092. Human, NM_203370. Zebrafish inca1, CK18555; inca2, BM071087. Human Inca-r, BC031558. Mouse Inca-r, NM_175398. Zebrafish inca-r, B885065. Xenopus (tropicalis) Inca-r, CR761080. chr, chromosome number.

Conserved requirements for Inca in NC development

To investigate the developmental requirements for Inca, MOs were designed to inhibit translation in both Xenopus and zebrafish embryos. These gave similar phenotypes. Two pseudoallelic Inca mRNAs in Xenopus laevis required two Inca MOs, designated IncaA MO and IncaB (GenBank Accession Number, DQ993180) MO. Injection of 10 ng per embryo of either MO efficiently inhibited Inca expression, animal caps were isolated from Xenopus embryos. These gave similar phenotypes. Two pseudoallelic Inca MOs, designated IncaA MO and IncaB MO2) that recognize non-overlapping target sequences yielded indistinguishable phenotypes (Fig. 4H,I). Injection of an inca1 MO into zebrafish injected embryos triggers neural induction and demonstrated reduced inca1 expression, which was blocked by coinjection of dnTfp2a. A similar but less severe effect was observed in zebrafish lockjaw (low) mutants, which lack a functional Tfp2a and demonstrated reduced inca1 expression. low mutants have defects in the NC-derived craniofacial skeleton and pigmentation (Knight et al., 2003). This correlates with reduced inca1 expression at the 10-somite stage in premigratory NC (Fig. 3B-F) and at 36 hpf (Fig. 3D,G).

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To determine whether Tfp2a is necessary and sufficient to activate Inca expression, animal caps were isolated from Xenopus embryos injected with one of three combinations of RNAs encoding: (1) Chd (at a dose lower than that of Fig. 1A) to trigger neural induction, (2) a mixture of Chd and Wnt3a at concentrations that induce NC, or (3) Chd, Wnt3a and a truncated, dominant negative Tfp2a (dnTfp2a) (Luo et al., 2002). Neural induction with Chd inhibited Inca expression compared with baseline levels in uninjected animal caps (which differentiate into epidermis), consistent with the lack of Inca expression in the neural plate in intact embryos (Fig. 3A). By contrast, NC induction by Chd+Wnt3a strongly activated Inca expression, which was blocked by coinjection of dnTfp2a. A similar but less severe effect was observed in zebrafish lockjaw (low) mutants, which lack a functional Tfp2a and demonstrated reduced inca1 expression. low mutants have defects in the NC-derived craniofacial skeleton and pigmentation (Knight et al., 2003). This correlates with reduced inca1 expression at the 10-somite stage in premigratory NC (Fig. 3B-F) and at 36 hpf (Fig. 3D,G).
caused a very similar, dose-dependent reduction in head size (Fig. 4J,K), as well as cranial cartilage formation in the pharyngeal arches (Fig. 4L,M) and neurocranium (Fig. 4N,O). To determine which NC cells require Inca and at what stages, we examined molecular markers of premigratory and migrating NC in MO-injected frog and fish embryos. Injection of 20 ng Inca MO into one cell at the two-cell stage caused no defects in expression of neural, epidermal or NC markers at early neurula stages (Fig. 5A-D) or in markers of migrating cranial NC at tailbud stages (data not shown). Both Dlx2 and Sox9 expression appeared slightly reduced as late as stage 32 (Fig. 5E-H). Nevertheless, cartilage development was severely impaired on the injected side (Fig. 5I). Similarly, Inca morphant zebrafish displayed mild reductions in expression of dlx2 and sox9a at 24 hpf, but severe reductions in the pharyngeal arches by 48 hpf (data not shown). These results suggest that cranial NC cells deficient in Inca form and migrate normally but fail to differentiate. TUNEL assays detected no differences in numbers of apoptotic cells in Inca MO and control MO-injected embryos at tailbud stages (Fig. 5K,L), but a significant increase by early tadpole stages, primarily in the eye and epidermis (Fig. 5M,N). By late tadpole stages, TUNEL signal was also detected in the jaw and dorsal cranium (Fig. 5O-R). All of these tissues derive from regions of earlier Inca expression (see Fig. 2).

These results in both fish and frog suggest that Inca is not required for NC induction or early migration, but later in cranial NC cells that form the head skeleton. To investigate requirements for Inca in NC morphogenesis at these later stages, we injected the inca1 MO into transgenic zebrafish that express eGFP under control of 5 kb of the
sox10 promoter (sox10:egfp), and used confocal microscopy to follow NC behaviors (Wada et al., 2005). Similar to inca1, sox10:egfp expression begins in premigratory cranial NC adjacent to the hindbrain during early somitogenesis, and persists in migrating NC cells (Fig. 5S,U), and craniofacial cartilage in the larvae (Fig. 5W,Y). With injection of 10 ng inca1 MO per embryo, NC cells migrated into the pharyngeal arches (Fig. 5T,V) but failed to condense and extend anteriorly to form normal cartilage (Fig. 5T,V). These results demonstrate conserved requirements for Inca in NC cells after they migrate.

**Inca interacts with PAK5**

Because Inca shows no sequence similarity to other gene families, we performed a yeast two-hybrid screen to identify potential binding partners that might link Inca to one or more cellular processes. Mouse Inca was used to screen a library of cDNAs from mouse embryos at E11, when Inca is abundantly expressed. Among several candidates, we repeatedly isolated the p21-activated kinase 4 (PAK4). Several truncated cDNAs isolated in this screen indicate that Inca binds to the C-terminal half of PAK4, which contains the kinase domain. *Xenopus* PAK5, a close relative of mammalian PAK4 (Cau et al., 2001), was similarly cotransfected into yeast with *Xenopus* Inca, and this also allowed robust growth on selective media, indicating similar physical interactions between Inca and PAK4/5 conserved from frog to mouse (Fig. 6A). This interaction was independently confirmed by co-immunoprecipitation (co-IP) of epitope-tagged *Xenopus* Inca and PAK5 co-expressed in HEK293 cells (Fig. 6B).

Both the two-hybrid and co-IP experiments involve expressing exogenous Inca and PAK4/5 at levels that may exceed physiologically meaningful concentrations, allowing spurious binding. To address this issue, antiserum directed against a synthetic peptide from *Xenopus* Inca and shown by western blotting to be specific for this protein (Fig. 6C), along with preimmune serum as a negative control, were used to immunoprecipitate proteins from an extract of *Xenopus* A6 cells, which express both PAK5 and Inca. Western blotting using an antibody for PAK5 showed that endogenous Inca protein will co-IP with PAK5 (Fig. 6D). Therefore, Inca and PAK5 naturally exist as a complex in untransfected *Xenopus* cells.

PAK proteins are effectors of the Rho GTPases Rac1 and Cdc42, and have been implicated in control of cytoskeletal dynamics, including microfilament and microtubule polymerization and stability (Bokoch, 2003; Jaffer and Chernoff, 2002). PAK5 interacts with both microfilaments and microtubules in the *Xenopus* embryo, and interference with PAK5 function affects cell adhesion and convergent extension movements, both of which depend upon cytoskeletal integrity and Rho GTPase signaling (Faure et al., 2005; Kofron et al., 2002; Wunnenberg-Stamp et al., 1999). To determine whether the Inca-PAK5 complex is also associated with the cytoskeleton, CHO cells were transiently cotransfected with Inca fused to GFP and PAK5 fused to red fluorescent protein (RFP) and observed with a confocal microscope. As shown in Fig. 6E, Inca and PAK5 colocalized in punctate bodies and fibers. Some of the endogenous PAK5 distribution pattern is in similar punctate bodies (Cau et al., 2001), but could also be due in part to overexpression artifacts. The fibers are likely to be microtubules, because they are sensitive to nocodazole (Fig. 6F).

**Ectopic expression of Inca modifies cytoarchitecture**

To determine whether misexpression of Inca outside of its normal domains in the NC and epidermis can alter cellular morphogenesis, synthetic IncaA mRNA was injected into *Xenopus* embryos at the one-cell stage. This disrupted body shape, including a shortened anteroposterior axis, open neural tube and multiple tissue protrusions (Fig. 7A,B). At blastula stages, pigment granules in individual blastomeres of Inca-injected embryos redistributed to the cell periphery, accenting cell-cell boundaries (Fig. 7C,D). These changes suggest a disruption in the cortical actin cytoskeleton in which these granules are embedded.

Inca misexpression also disrupted another process dependent on cytoskeletal rearrangements in *Xenopus*, wound healing. In low to moderate salt concentrations, an embryo from which an ectodermal explant was excised (vegetal explant) healed within approximately 15-20 minutes. This involves changes in cell movements and adhesion that depend on Rho GTPase signaling, plakoglobin and other components (Davidson et al., 2002; Tao et al., 2005). Vegetal explants from embryos injected with Inca mRNA healed much more slowly and to a lesser extent than uninjected controls (Fig. 7E,F). Healing explants normally formed a ‘purse string’ of actin filaments at the edge of the wound a few minutes after excision from the blastula (Merriam and Christensen, 1983), which is under the control of the Rho GTPases Rac1 (Brock et al., 1996) or Cdc42 (Kofron et al., 2002). Phalloidin Fig. 3. Inca expression in NC depends on Tpap2a. (A) Northern analysis of animal cap RNA from embryos injected with Chd, Chd+Wnt3a, or Chd+Wnt3a+dnTpap2a (Chd+Wnt+ΔAD) and probed for *Xenopus* Inca, with 18S rRNA as a loading control. Expression induced by Chd+Wnt is blocked by dnTpap2a. (B-G) Whole-mount in situ hybridizations showing zebrafish inca1 expression in wild type (B-D) and low mutants (E-G). B,E are lateral views and C,F dorsal views at the 10-somite stage. D,G are lateral views at 36 hpf. Numbers indicate presumptive pharyngeal arches. oe, olfactory epithelia.
staining never detected purse-string structures in explants from embryos injected with Inca mRNA (Fig. 7G,H). Thus, ectopic Inca in the blastula disrupts multiple cell behaviors dependent on rearrangements of cortical actin.

To test possible roles for Inca-PAK5 interactions in wound healing, RNAs encoding Inca, and PAK5, as well as a kinase-dead form of PAK5 (PAK5/KR) and a constitutively active form (PAK5/EN), all as eGFP fusions (Cau et al., 2001), were injected individually and in combination into Xenopus embryos. Vegetal explants derived from these embryos were cultured for 25 minutes and examined for wound closure. Whereas expression of Inca inhibited healing (Fig. 7M), wild-type PAK5 and PAK5/KR injections had minimal effects (Fig. 7J,K). PAK5/EN injections resulted in some inhibition (Fig. 7L), but less than observed with Inca. By contrast, when Inca and wild-type PAK5 were co-injected, there was a dramatic failure of wounds to heal, accompanied by extensive loss of cell adhesion (Fig. 7N). This effect depended upon the kinase activity of PAK5, because co-injection of Inca and PAK5/KR had a much weaker effect compared with co-injection of Inca and wild-type PAK5 (Fig. 7O). Inca- and PAK5-eGFP fusions allowed simultaneous monitoring of protein levels using anti-GFP antibody (Fig. 7P).

PAK5 protein levels were comparable, as were the Inca levels, but in significant molar excess over Inca. The contrast in phenotype between PAK5/EN and PAK5 overexpression with Inca suggests that the PAK-Inca synergism is not because of activation of the PAK5 kinase by Inca. Further evidence for this is that Inca-PAK5 interaction does not enhance PAK5 kinase activity in cotransfected HEK293 cells (Fig. 7Q). PAK5 expression is widespread during early and late development, overlapping with Inca, so both proteins are likely to be present in NC from early stages onward (Fig. 7R). These results indicate that the interaction of Inca and PAK5 has profound effects on adhesive and cytoskeletal functions.

**DISCUSSION**

In view of the essential functions of Tfap2a in embryonic vertebrate development, it is important to identify genes that lie downstream from this transcriptional activator. Using a microarray screen based on inducible Tfap2a (Luo et al., 2005), we have identified a novel protein, Inca, required for NC development in both Xenopus and zebrafish. Inca interacts with PAK5 to control cytoskeletal rearrangements in the early embryo. Our results lead us to propose that Inca-PAK5 interactions mediate Tfap2a-dependent NC cell behaviors at least in part by regulating their cytoskeleton.

Our screen for Tfap2a targets has also yielded a novel protocadherin, named PCNS, that is required for NC migration (Rangarajan et al., 2006), and a surprisingly high proportion of epidermal genes also expressed in the NC (Luo et al., 2005). From these results and earlier work, a picture emerges in which Tfap2a
mediates BMP signaling, functioning in concert with and downstream of other transcription factors that initiate NC and epidermal specification. In the case of cranial NC, however, Tfap2a appears more important as an early regulator of other ‘effector’ genes that control cell specification and terminal differentiation (Meulemans and Bronner-Fraser, 2004). In the NC, these include Tfap2a-dependent genes such as Inca, PCNS, Hoxa2 and C-kit, all of which may control certain aspects of cellular morphogenesis. For example, suppression of both TFAP2A and C-KIT in humans correlates with an invasive, metastatic melanoma phenotype (Huang et al., 1998).

Inca regulates craniofacial cartilage morphogenesis

Knockdown of Inca expression with morpholinos has equivalent endpoints – craniofacial cartilage hypoplasia – in both Xenopus and zebrafish embryos. Controls strongly argue against MO toxicity or off-target effects. Inca morphants show no defects in gene expression during NC induction or early migration, but only later as chondrocytes differentiate. In zebrafish, chondrocyte precursors in the NC condense into cartilage primordia and then stack into linear arrays (Kimmel et al., 2001). Our analysis of sox10:egfp has revealed that NC cells migrate into the arches successfully in Inca morphants but fail to condense or organize into stacks, and eventually begin to undergo apoptosis.

Given the early requirements for Tfap2a in NC and the early NC expression of Inca, this relatively late phenotype is unexpected. The skeletal defects seen in low embryos are not as severe as in inca1 morphants, which is consistent with the observed reduction, but not complete loss, of inca1 expression in NC cells that form craniofacial cartilage in low embryos. We argue that the relatively late phenotype reflects requirements for Inca in cytoskeletal regulation in NC cells later in migration. Although morphants may retain some low-level Inca expression masking a complete null phenotype, we have injected much higher doses of Inca MO in Xenopus embryos (up to 80 ng per embryo; data not shown), which gives an identical phenotype. Alternatively, Inca could share redundant functions with other proteins during early NC formation and migration. Vertebrates have an Inca-related gene (Inca-r), but our preliminary analyses suggest that this gene is not expressed in early development (data not shown).

Defects in NC morphogenesis in the absence of Inca function coincide with elevated cell death in tissues that express Inca, including the eye, the epidermis and the NC. By the tadpole stage, cranial cartilage was eliminated on the injected side (left side in I). Control MO injection had no effect (J). (K-R) TUNEL staining of embryos injected with control MO (K,M,O) or Inca MO (L,N,P-R), cultured to stage 21 (K,L), stage 30 (M,N) or stage 45 (O-R). Q and R are dorsal and ventral views, respectively, of the tadpole shown in P. Black arrowheads indicate strong TUNEL signal in neurocranium (Q) and jaw cartilage (R). (S-Z) Confocal images of sox10:eGfp expression in cranial NC cells of controls (S,U,W,Y) and inca1 morphants (T,V,X,Z). Lateral views, anterior to the left. Stages are indicated in the panels. First and second pharyngeal arches are indicated in S,T, and derived cartilage elements by arrowheads in Y,Z. Asterisks indicate expression of sox10:eGFP in the otic vesicle.
Inca modulates cytoskeletal dynamics in association with PAK5

Ectopic overexpression of Inca causes defects in *Xenopus* blastula-stage embryos that occur much earlier than the loss-of-function phenotypes, probably because of disruption of the microfilament cytoskeleton. Cortical pigment becomes redistributed away from the apical surface of Inca-injected blastomeres. These pigment granules depend specifically on F-actin, as shown by treatment with cytochalasin B, and show no response to anti-microtubule drugs such as taxol or nocodazole (Moreau et al., 1999). The Inca-induced pigment redistribution resembles embryos overexpressing FRIED, a protein tyrosine phosphatase that interacts with Frizzled-8 (Itoh et al., 2005). Activated GTPase Rac1 rescues effects of FRIED overexpression, consistent with the hypothesis that this restores cortical actin organization. Likewise, ectodermal wound healing in *Xenopus* embryos depends on restructuring of the cortical actin cytoskeleton. This is regulated by the small GTPase Cdc42 (Kofron et al., 2002), and Inca overexpression interferes with assembly of the actin purse-string around wounds. These results suggest that Inca might be involved in the regulation of the Rho GTPase signaling pathway.

The PAKs are serine-threonine protein kinases that associate with Rac and Cdc42. *Xenopus* PAK5 is expressed maternally and, by the gastrula stages, expression overlaps that of Inca in the ectoderm, although it is not restricted to the inner, sensorial layer (Faure et al., 2005). At later stages, PAK5 expression appears ubiquitous (Fig. 7R). Our results suggest that the tissue-specific distribution of Inca and PAK5, particularly their co-expression in ectoderm, has a significant impact on cytoskeletal organization.

PAK5 localizes to microtubules and actin filaments, in patterns that shift during the cell cycle and do not depend on kinase activity. Constitutive activation of PAK5 prevents its interactions with microtubules (Cau et al., 2001). However, PAK5 kinase activity plays an essential role in early *Xenopus* gastrulation, because overexpression of a kinase-dead mutant form (PAK5/KR) acts as a dominant negative to inhibit convergent extension movements in dorsal mesoderm (Faure et al., 2005). PAK5/KR overexpression also enhances calcium-dependent adhesion of ectodermal cells, whereas a constitutively active kinase reduces adhesion. Inca synergizes with an intact PAK5 to disrupt wound healing and cell adhesion when overexpressed in *Xenopus* embryos. PAK5/KR shows no synergy, suggesting that this interaction requires kinase activity (Fig. 7). However, Inca does not activate the PAK5 kinase, because co-expression of *Xenopus* Inca and PAK5 in mammalian cells does not increase phosphorylation of the regulatory Ser533 residue (Fig. 7Q). Furthermore, overexpression of constitutively active PAK5 does not mimic combined overexpression of Inca and wild-type PAK5. This suggests the possibility that kinase targets for PAK5 may exist in microtubule or microfilament cytoskeletal components that are only
accessible when Inca is associated with the kinase. In other words, Inca might provide a mechanism for regulation of PAK5, which, like other Class II PAKs (PAK 4-6), binds to Cdc42 or Rac1 but is not catalytically activated by this association.

Interactions of Inca with PAK5 and the cytoskeleton may also help explain why Inca morphants have defects in NC morphogenesis. Requirements for PAK4/5 in later embryogenesis remains unclear because dominant-negative PAK5 in *Xenopus* disrupts gastrulation, pigmentation and wound healing in *Xenopus*. (A) Uninjected stage 25 control embryo. (B) Siblings injected with 250 pg IncaA mRNA have shorter body axes and numerous protruberances. (C,D) High-magnification views of ectoderm at stage 8 from un.injected (C) and IncaA mRNA-injected (250 pg; D) embryos showing relocation of cortical pigment to the cell periphery. Scale bar: 100 µm. (E,F) Vegetal explants after animal cap removal and culture for 5 (E) or 25 (F) minutes in 0.3×MMR. Upper row, un.injected; lower row injected with 250 pg Inca mRNA, showing delayed healing. (G,H) Confocal z-stack images of animal caps from un.injected (G) and IncaA mRNA-injected (H) embryos after 5 minutes of healing in 0.3×MMR. White arrowheads in G indicate purse-string actin-filament structures missing in embryos overexpressing Inca. (I-O) Synergistic effect on wound healing of PAK5 and Inca. Embryos injected with mRNA encoding wild-type PAK5-GFP (J), kinase-dead PAK5-GFP (PAK5/KR; K), constitutively active PAK5-GFP (PAK5/EN; L), IncaA-GFP (M), a mixture of IncaA-GFP and PAK5-GFP (N) or a mixture of IncaA-GFP and PAK5/KR-GFP (O). All PAK5-GFP mRNAs injected at 1 ng, IncaA-GFP mRNA at 250 pg. Vegetal explants were allowed to heal for 25 minutes. Combining Inca-wild-type PAK5 greatly delays wound healing and leads to dissociation (N). PAK5/KR does not exhibit this synergistic effect (O). (P) Uninjected control embryo. Results from these experiments are summarized in Table 1. (P) PAK5-GFP mRNAs were equally translated, as judged by western blot using antibody to GFP. (Q) Co-expression with Inca does not increase PAK5 phosphorylation. Western blots of extracts made from HEK293 cells transfected with 0.5 µg FLAG-tagged XPAK5 plasmid and 0, 0.5 or 1.0 µg of Myc-tagged *Xenopus* Inca. The ratio of total XPAK5 signal (FLAG antibody) to the level of phosphorylation of regulatory serine residue 474 (phospho-PAK4) was not significantly different in any of the samples. (R) *Xenopus* PAK5 expression. Whole-mount in situ hybridization at stages 10, 13 and 25. Stage 10 embryos were bisected in the sagittal plane prior to hybridization. PAK5 expression is broad in ectoderm and mesoderm at early stages and essentially ubiquitous later in development.

### Table 1. Summary of data from Fig. 7

<table>
<thead>
<tr>
<th>RNA</th>
<th>Amount injected</th>
<th>Total number of embryos (n)</th>
<th>Percentage with phenotype (%)</th>
<th>Phenotype</th>
<th>Part</th>
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<tr>
<td>IncaA</td>
<td>250 pg</td>
<td>84</td>
<td>95</td>
<td>Short axis, abnormal shape</td>
<td>B</td>
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<tr>
<td></td>
<td>250 pg</td>
<td>126</td>
<td>100</td>
<td>Cortical pigment dispersal</td>
<td>D</td>
</tr>
<tr>
<td></td>
<td>250 pg</td>
<td>48</td>
<td>100</td>
<td>Inhibited wound healing</td>
<td>E,F</td>
</tr>
<tr>
<td>PAK5</td>
<td>1 ng</td>
<td>24</td>
<td>100</td>
<td>Normal wound healing</td>
<td>J</td>
</tr>
<tr>
<td>PAK5/KR</td>
<td>1 ng</td>
<td>24</td>
<td>100</td>
<td>Normal wound healing</td>
<td>K</td>
</tr>
<tr>
<td>PAK5/EN</td>
<td>1 ng</td>
<td>24</td>
<td>100</td>
<td>Inhibited wound healing (minor)</td>
<td>L</td>
</tr>
<tr>
<td>IncaA</td>
<td>250 pg</td>
<td>24</td>
<td>100</td>
<td>Inhibited wound healing (moderate)</td>
<td>M</td>
</tr>
<tr>
<td>IncaA+PAK5</td>
<td>250 pg+1 ng</td>
<td>24</td>
<td>100</td>
<td>Inhibited wound healing (severe)</td>
<td>N</td>
</tr>
<tr>
<td>IncaA+PAK5/KR</td>
<td>250 pg+1 ng</td>
<td>24</td>
<td>100</td>
<td>Inhibited wound healing (moderate)</td>
<td>O</td>
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gastriation (for a review, see Nikolaidou and Barrett, 2005). It is attractive to hypothesize that skeletogenic NC cells require Inca to control PAKS-dependent cytoskeletal restructuring and NC cell behaviors that are essential for proper craniofacial morphogenesis.

Special thanks to Nathalie Morin for generously providing Xenopus PAK plasmids and antibody, and to Maria Morasso for mouse embryo samples and northern blot. Thanks to Vincent Shram at the NICHD Microscopy and Imaging Core Facility for assistance with Xenopus confocal microscopy. This work was supported in part by the Intramural Research Program of the National Institute of Child Health and Human Development, NIH, and by grants from the NIH (DE-13828, NS-41533) and March of Dimes (1-FY01-98) to T.S. TLH. was supported by a postdoctoral fellowship from the American Cancer Society.

References


