Introduction

Cadherins comprise a superfamily of cell-cell adhesion molecules that are involved in intercellular adhesion in a wide variety of cell types. Modulation of the strength of cell-cell adhesion is essential during morphogenetic processes involving cell movements and tissue rearrangements. Functional inactivation of cadherins during these events seems to be an important step in the regulation of the balance between cell adhesion and motility (Gumbiner, 2000; Perez-Moreno et al., 2003; Tepass et al., 2000). Dysfunction of this balance is often observed in cancer cells, where the loss of E-cadherin expression and disruption of cell-cell adhesion correlates with tumor invasion (Mareel and Leroy, 2003). While physical interaction between two cadherins is a prerequisite for intercellular adhesion, it is clearly not of sufficient force. Adhesive strength is considerably enhanced by clustering of the adhesion molecules in the membrane, and by their physical interaction with the cytoskeleton (Gumbiner, 2000). Interestingly, cadherins are also capable of signaling to the cytoplasm (Yap and Kovacs, 2003; Braga, 2002). These signals can change the behavior of the cell and the organization of the cytoskeleton. Hence, one can envision a scenario in which the homophilic interaction between cadherins on neighboring cells not only provides physical adhesion, but also locks the cells in an immobile condition via signaling.

Cadherins are associated in the cytoplasm with catenins, which link them to the cytoskeleton and can regulate their adhesive functions. The highly conserved distal region of the cytoplasmic tail of cadherin binds β-catenin or plakoglobin (γ-catenin). These proteins can interact with α-catenin, which provides a direct or indirect link with the actin cytoskeleton, a link that is required for development of strong adhesion. The second highly conserved region of the cadherin cytoplasmic tail is localized near the membrane, and binds several p120 catenin family members, including p120 catenin (p120), ARVCF and δ-catenin/NPRAP. This domain was also identified as a binding site for the ubiquitin-ligase Hakai and presenilin 1, both of which may be involved in the trafficking/recycling of cadherins (Fujita et al., 2002; Uemura et al., 2003; Marambaud et al., 2002; Baki et al., 2001). Functional analysis of the cytoplasmic domain of E- and N-cadherin identified the juxtamembrane domain (JMD) as a domain interfering with cell motility/invasion, neuronal outgrowth and ectodermal cell adhesion (Chen, 1997; Kintner, 1992; Riehl, 1996). Other studies implicated the JMD in positive and/or negative regulation of cell adhesion (reviewed during Xenopus development, p120 transcripts are enriched in highly morphogenetic tissues. We addressed the developmental function of p120 by knockdown experiments and by expressing E-cadherin mutants unable to bind p120. This resulted in defective eye formation and provoked malformations in the craniofacial cartilage structures, derivatives of the cranial neural crest cells. Closer inspection showed that p120 depletion impaired evagination of the optic vesicles and migration of cranial neural crest cells from the neural tube into the branchial arches. These morphogenetic processes were also affected by p120-uncoupled cadherins or E-cadherin containing a deletion of the juxtamembrane domain. Irrespective of the manipulation that caused the malformations, coexpression of dominant-negative forms of either Rac1 or LIM kinase rescued the phenotypes. Wild-type RhoA and constitutively active Rho kinase caused partial rescue. Our results indicate that, in contrast to invertebrates, p120 is an essential factor for vertebrate development and an adequate balance between cadherin activity and cytoskeletal condition is critical for correct morphogenetic movements.

Summary

During Xenopus development, p120 transcripts are enriched in highly morphogenetic tissues. We addressed the developmental function of p120 by knockdown experiments and by expressing E-cadherin mutants unable to bind p120. This resulted in defective eye formation and provoked malformations in the craniofacial cartilage structures, derivatives of the cranial neural crest cells. Closer inspection showed that p120 depletion impaired evagination of the optic vesicles and migration of cranial neural crest cells from the neural tube into the branchial arches. These morphogenetic processes were also affected by p120-uncoupled cadherins or E-cadherin containing a deletion of the juxtamembrane domain. Irrespective of the manipulation that caused the malformations, coexpression of dominant-negative forms of either Rac1 or LIM kinase rescued the phenotypes. Wild-type RhoA and constitutively active Rho kinase caused partial rescue. Our results indicate that, in contrast to invertebrates, p120 is an essential factor for vertebrate development and an adequate balance between cadherin activity and cytoskeletal condition is critical for correct morphogenetic movements.

Supplemental data available online

Key words: Embryogenesis, Cadherins, Catenins, Rho GTPases, Xenopus
by Anastasiadis and Reynolds, 2000). Emerging evidence suggests that p120 is a potential regulator of the adhesive strength of cadherins. Whereas some studies demonstrated a positive role for p120 in the dimerization and clustering of cadherins in the plasma membrane and in cell-cell junction formation, others indicated that p120 has a negative effect on cadherin-mediated cell-cell adhesion. In an effort to unify these conflicting data, a model has been proposed in which post-translational modifications of p120, probably including phosphorylations, lead to its ‘activation’ or ‘inactivation’ (Anastasiadis and Reynolds, 2000). In this model, p120 acts as a switch that can promote either strong or weak adhesion. Surprisingly, experiments in Drosophila have indicated that a p120-uncoupled DE-cadherin mutant can act as a perfect substitute for wild-type DE-cadherin, making it unlikely in this system that the binding of p120 is important for the functional regulation of DE-cadherin (Pacquelet et al., 2003). Very recent experiments on mammalian cell lines demonstrated that p120, in addition to influencing cadherin function directly, influences the stabilization and recycling of cadherin/catenin complexes at the plasma membrane (Chen et al., 2003; Davis et al., 2003; Xiao et al., 2003). Moreover, it has been found that p120 binds to kinesin and microtubules and hence may assist in targeting of cadherin/catenin complexes to sites of cell-cell contact (Chen et al., 2003; Franz and Ridley, 2003).

p120 has been shown to modulate the activity of Rho GTPases, leading to morphological changes and increased cell migration (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). The Rho GTPases represent a highly conserved family of molecules that regulate a variety of cellular functions, including the dynamics of the actin cytoskeleton, cell adhesion, transcription, cell growth and membrane trafficking (reviewed by Etienne-Manneville and Hall, 2002). The mechanism by which p120 regulates the Rho GTPases is still not well understood, but it has been proposed that it involves direct interaction of p120 with RhoA or Vav2, which is a guanine nucleotide exchange factor (GEF) for Rac1, Cdc42 and RhoA (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). The ability of Rho family members to regulate the adhesive and motile behavior of cells makes them good candidates for involvement in morphogenetic processes during embryonic development, where precise shape changes, tissue rearrangements and cell movements are required. Substantial information regarding the function of Rho GTPases has been derived from studies in Drosophila, but recent evidence suggests that Rho proteins are important for morphogenesis in vertebrates as well (Van Aelst and Symons, 2002).

The function of p120 during development has recently been addressed in Drosophila. Null alleles for p120 look phenotypically normal, but when analyzed in crosses, the p120 deficiency enhances the phenotypes of DE-cadherin and Armadillo mutants (Myster et al., 2003). Comparable results were obtained for the Caenorhabditis elegans ortholog of p120, JAC-1 (Pettitt et al., 2003). Furthermore, it has been reported that Rho1, the Drosophila ortholog of RhoA, interacts physically and functionally with p120 (Magie et al., 2002). The role of p120 in vertebrate development was analyzed by overexpression and recently also by morpholino-mediated depletion in Xenopus embryos (Geis et al., 1998; Paulson et al., 1999; Fang et al., 2004). While overexpression of p120 in the dorsal mesoderm induced severe gastrulation defects, the depletion of p120 in addition affected convergent extension movements. Interestingly, in agreement with reports in cell culture, the effects of p120 depletion could be rescued by co-injection of dominant negative RhoA and dominant active Rac (Fang et al., 2004).

In this study we wanted to explore the role of p120 in vertebrate development. We report the cloning of the Xenopus p120 ortholog and analysis of the expression pattern in the early embryo. We found that p120 transcripts are highly enriched in the eye vesicles and in the branchial arches. Consequently, we investigated the morphogenetic repercussions induced in Xenopus embryos by depletion of p120, and by expression of point and deletion mutants of E-cadherin that are unable to bind p120. We targeted our injections to a region contributing mainly to the (neuro)ectodermal tissues of the future head region so that we would not interfere with gastrulation and convergent extension movements. Depletion of p120 and overexpression of the E-cadherin mutants induced perturbations in the developing eyes and in the craniofacial cartilage skeleton, a derivative of the cranial neural crest. We also demonstrate that dominant negative forms of Rac and LIM kinase, and to a lesser extent wild-type RhoA and constitutively active Rho kinase, rescue the phenotypes induced by p120 depletion or ectopic mutant cadherins. Our results indicate that p120 is crucially involved in regulating morphogenetic cell movements in the anterior region of early embryo, most likely by modifying the activity of the cadherins at the membrane, and coordinating their functional interactions with the cytoskeleton.

**Materials and Methods**

Reverse transcription and PCR

Total RNA was extracted from XTC cells, Xenopus adult tissues and embryos from stage 0 to stage 39 using RNAzol (Wak-Chemie Medical, GMBH) according to the manufacturer’s instructions. To detect Xp120 transcripts, cDNA was generated from 1 μg of total RNA using Superscript reverse transcriptase (Life Technologies) as described previously (Keirsebilck et al., 1998). cDNA (3 μl) was amplified using Taq polymerase (Boehringer Mannheim) or Pfu polymerase and the primers 5’ TAGAGGCGCTGGAAGTGTGA 3’ and 5’ ATCAGTTGGCATTGTGAATC 3’.

**Plasmid constructs**

To generate plasmids for RNA injection, the cDNAs encoding the Xp120 isoforms were amplified from stage 30 Xenopus embryos using Pfu DNA polymerase, with primers incorporating EcoRI (5’ end) and SpeI (3’ end) restriction sites. Amplified fragments were sequenced and cloned in the EcoRI-XbaI sites of pCS2+ (Turner and Weintraub, 1994). The full-length mouse E-cadherin (mE-cad) cDNA was isolated from the pBATEM2 plasmid by digestion with BglII and XhoI and subcloned in the BamHI and XbaI sites of the pCS2+ vector. The mE-cad cDNA sequence in between the coding regions of the cadherins was amplified using Taq polymerase (Boehringer Mannheim) or Pfu polymerase and the primers 5’ TAGAGGCGCTGGAAGTGTGA 3’ and 5’ ATCAGTTGGCATTGTGAATC 3’.

The plasmid pSp64T-XEcad, carrying the full-length Xenopus E-cadherin (XE-cad) cDNA, was digested with ClaI and XhoI, and the fragment corresponding to the full-length XE-cad cDNA was cloned into the corresponding sites of the pCS2+ vector. The pCS2+XEcad...
plasmid was used in site directed mutagenesis (Stratagene) to generate the p120-uncoupled XE-cadh750AAA and the XE-cadh753AAA mutants. For in vitro transcription, the full length human WT-RhoA, CA-RhoAV14, DN-RhoAV19, CA-Rac1V12 and DN-Rac1V12N17 were subcloned from the pcDNA3.1 vector into the pCS2+ plasmid, and CA-ROCK was subcloned from pEF-bos-myc into the pCS2+plasmid.

RNA and morpholino injections
For RNA injections, all constructs were linearized with NotI, except for WT/XLKI and WT/XLKI(KD) (encoding WT and DN LIMK), which were linearized with EcoRI. Capped mRNAs were synthesized in vitro from linearized plasmids using SP6 polymerase (Promega). Integrity of RNA was checked on denaturing agarose gels. The morpholinos used were p120 MO 5’ ACTCTGGCTCACCT-ATATAGAAAAGG 3’ and a standard control oligo as negative control (Gene Tools).

Xenopus eggs were fertilized by standard methods (Newport and Kirschner, 1982), dejellied with 2% cysteine (pH 8.0), rinsed and incubated in 0.1x MMR (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO4, 0.2 mM CaCl2, 0.5 mM Hepes, pH 7.4). Embryos were placed in 1x MMR containing 6% Ficoll, and injected with mRNA. Two hours after injection, embryos were transferred to 0.1x MMR and cultured at 17°C until they had reached the neurula stage. Stages of embryonic development were determined according to a published table of Xenopus laevis development (Nieuwkoop and Faber, 1994).

Cell tracing in embryos
For the tracing experiments with Rhodamine, embryos were injected in 1 cell at the 32-cell stage with dextran, tetramethylRhodamine (Xenopus laevis development were determined according to a published table of MMR containing 6% Ficoll, and injected with mRNA. Two hours

DNB-caged Fluorescein, $M_r$ 10×10^3, anionic (Microleap Bases) in combination with morpholinor or RNA. Embryos were monitored with a Leica MZFLIII fluorescence stereomicroscope, and pictures were taken with a Leica DC 300F camera. Desired stages were fixed for 1 hour in MEMFA at room temperature, embryos were washed 3 times in PBS and soaked overnight in 20% sucrose/PBS. Embryos were embedded in cryo-embding compound (Microm). Sections of 12 μm were cut. Sections were dried at room temperature and mounted with Vectashield containing DAPI (VECTOR laboratories). Images were obtained with an Axiophot microscope (Carl Zeiss) using a 10× objective equipped with a digital camera (MicroMAX, RS Photometrics), and processed using Metamorph Image software (Universal Imaging).

Immunofluorescence and confocal microscopy
For immunohistological staining of sections, embryos were fixed in DMSO/methanol overnight at –20°C, rinsed in PBS and soaked for 24 hours in 15% gelatin/15% sucrose. Afterwards embryos were embedded in cryo-embedding compound (Microm). Sections of 12 μm were cut. Sections were dried for 1 hour at room temperature, dipped for 2 minutes in acetone, rehydrated in PBS, washed for 20 minutes in PBS/Tween and incubated with the primary antibody (Decma-1, Sigma, 1:1000) overnight at 4°C. After incubation with the primary antibody, sections were washed 3 times with PBS/Tween and incubated with the secondary antibody (anti-rat Alexa 488, 1:500) for 2 hours at 30°C. Afterwards sections were washed 3 times with PBS/Tween and incubated in DAPI/PBS for 15 minutes. Sections were washed again 2 times with PBS and incubated in PBS containing 0.1% Eriochrome Black for 5-15 minutes (Sigma) to mask autofluorescence. Sections were mounted with vextashield (VECTOR laboratories). Images were obtained with an Axiophot microscope (Carl Zeiss) using a 20× objective; the microscope was equipped with a digital camera (MicroMAX, RS Photometrics) and Metamorph Image software (Universal Imaging). The sections were also examined with an inverted confocal Laser Scan Microscope 410 (Carl Zeiss) using an Argon laser (488 nm) and appropriate settings. The confocal images in Fig. 10B were selected from the 19 images made by z-sectioning (1 μm Z-spacing) on living embryos.

Results
Isolation of the Xenopus p120 ortholog
We isolated sequences encoding the Xenopus p120 ortholog in a yeast two-hybrid screening, using the cytoplasmic tail of Xenopus N-cadherin lacking the β-catenin binding site as bait. The merged cDNA sequences indicated two open reading frames of 2577 and 2355 bp. Alignment of the deduced amino acid sequence of the longer open reading frame with the human p120 isoform 1ABC revealed 48% identity and 62% similarity (Fig. 1A). Sequence alignment with other p120 family members indicates that the isolated clones indeed represent the Xenopus p120 ortholog (Table 1). Particularly high sequence conservation was observed in the armadillo domain and within the N-terminal coiled-coil region, with less sequence homology present at the C- and remaining N-terminal regions. Sequence

washes in ethanol/PBS, and transferred to JB-4 embedding medium (Polysciences). Sections of 5 μm were made with a Universal Heavy Duty Microtome and stained with Hematoxilin and Eosin. Alcian Blue staining was performed as described (Pasqualetti et al., 2000).

Immunoblotting
For western blot detection, injected embryos were collected at different time points, lyzed in NP-40 lysis buffer (0.5% NP-40, 150 mM NaCl, 50 mM Tris pH 7.5, 2 mM EDTA, 0.02% NaN3) and cleared by centrifugation for 5 minutes at 4°C. Laemmli sample buffer was added to the supernatants and the equivalent of three embryos was loaded per lane and separated in an 8% SDS-polyacrylamide gel. The proteins were blotted onto a nitrocellulose membrane and detected with polyclonal anti-Xp120, anti-C-cadherin, mouse monoclonal anti-E-cadherin, mouse monoclonal anti-Myc tag 9E12, rat monoclonal anti-N-cadherin (Zymed), and rat monoclonal anti-Mβ-cadherin ECD-2 (Sigma). After washing, blots were incubated with HRP-conjugated anti-rabbit, anti-mouse and anti-rat IgGs, respectively (Amersham Pharmacia Biotech), followed by washing and developing with enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

Whole-mount in situ hybridization and Alcian Blue staining
A plasmid containing full-length Xpl20isos1 cDNA was linearized with Ndel or SnaBI and used to prepare sense and antisense riboprobes, which were digoxigenin-labeled according to the manufacturer’s instructions (Boehringer Mannheim). Whole-mount in situ hybridization and sectioning of stained specimens were performed as described (Bellefroid et al., 1996). Embryos were bleached for 1-2 hours under UV in Mayor bleaching solution (1% H2O2, 5% formamide, 0.5× SSC) and incubated in 2:1 (v/v) benzyl benzoate/benzyl alcohol clearing solution for inspection with a stereomicroscope (Carl Zeiss). For histological analysis, embryos were cleared by centrifugation for 5 minutes at 4°C. Sections were washed again 2 times with PBS and incubated in PBS containing 0.1% Eriochrome Black for 5-15 minutes (Sigma) to mask autofluorescence. Sections were mounted with vectashield (VECTOR laboratories). Images were obtained with an Axiophot microscope (Carl Zeiss) using a 20× objective; the microscope was equipped with a digital camera (MicroMAX, RS Photometrics) and Metamorph Image software (Universal Imaging). The sections were also examined with an inverted confocal Laser Scan Microscope 410 (Carl Zeiss) using an Argon laser (488 nm) and appropriate settings. The confocal images in Fig. 10B were selected from the 19 images made by z-sectioning (1 μm Z-spacing) on living embryos.
analysis showed an alternatively spliced region of 210 nucleotides within the N-terminal region of Xp120, which removes sequences containing an upstream translational start site and directs the use of the second downstream translational start site. Using RT-PCR we confirmed that both p120 transcripts that were identified in the yeast two-hybrid screen are indeed present in Xenopus embryos (Fig. 1B) as well as in the XTC cell line (data not shown). In the adult frog, both p120 transcripts were detected in all tissues examined (Fig. 1C).

Although up to 48 splicing isoforms are predicted for human p120 (Aho et al., 1999; Keirsebilck et al., 1998), we were unable to detect any additional splicing events for Xenopus p120 (Xp120iso2). An RT-PCR reaction of elongation factor 1 (EF1a) was used as a loading control.

p120 mRNA is enriched in highly morphogenetic regions during Xenopus laevis development

To gain insight into the role of p120 during Xenopus development, we examined its spatio-temporal expression pattern by semi-quantitative RT-PCR and whole-mount in situ hybridization. Our results demonstrate that p120 is expressed both maternally and during all stages of early embryogenesis. Before gastrulation, the p120 transcript representing the shorter isoform appears to be more abundant (Fig. 1B). In early gastrula stage embryos, p120 is enriched in the animal hemisphere (Fig. 2a). In the late neurula, expression of p120 was enhanced in the anterior region, especially in the optic vesicles (Fig. 2b). At the tailbud stage, inspection of whole embryos (Fig. 2c) or transverse sections (Fig. 2d) indicated that p120 expression was clearly enriched in the head region, particularly in the optic vesicle, ear vesicle, olfactory placode and branchial arches. Weakly elevated expression of p120 was also detected in the somites, the notochord and the pronephros. In summary, while p120 is ubiquitously expressed during development, its levels are particularly high in tissues participating in prominent morphogenetic movements.

Depletion of p120 affects the eyes and the craniofacial skeleton

It was recently reported that depletion of p120 in whole Xenopus embryos results in severe gastrulation defects and interferes with convergent extension movements (Fang et al., 2004). Our in situ hybridization data, showing increased expression of p120 mRNA in certain tissues of the anterior...
region of the embryo, prompted us to determine whether p120 depletion can also interfere with the development of anterior (neuro)ectodermal structures. For this, we injected a morpholino (p120 MO) targeted to the start codon and the 5' UTR of p120 into the animal region of the two dorsal blastomeres of a 16-cell stage embryo. As a control, we injected a morpholino targeted to an irrelevant sequence (control MO). Dorsal-animal injection targets the region that contributes mainly to head ectodermal structures, such as the cranial neural crest, the retina, the lens, the olfactory placode and the brain (Moody, 1987). When embryos injected with p120 MO were scored at the tailbud or tadpole stages, we observed that eye formation was clearly affected (Fig. 3A, Table 2). In the majority of these embryos, the size of one or both eyes was reduced, or eyes were totally absent. Other anterior structures, such as the prosencephalon and the olfactory placodes and bulks, were morphologically normal. No phenotype was observed upon injection of up to 50 pg of control MO, and the p120 MO-induced eye phenotype could be rescued by co-injection of Xp120 mRNA (Table 2). Importantly, because our injections were targeted to the dorsal-animal region, the observed eye phenotype does not result from interference of p120 depletion with the activity of the Spemann Organizer and the anterior endomesoderm, two regions involved in patterning of the dorso-anterior axis. The injected region also does not participate in the convergent extension movements of the dorsal mesoderm or the neural ectoderm. Consequently, all injected embryos underwent normal gastrulation and axis elongation.

Interestingly, when p120 MO-injected embryos were grown until stage 45-46, we observed additional developmental abnormalities characterized by smaller craniofacial cartilage structures resulting in reduced width of the head (Fig. 3A and 3B). Staining of the cartilage with Alcian Blue showed that, despite the reduced size, there were no gross morphological abnormalities in the structures of those cartilages (see section on cadherin deletion mutant). The cartilage phenotype was not observed with control MO.

Depletion of p120 affects specific endogenous cadherin levels
Recent studies in cell lines have shown that binding of p120 may be important to stabilizing the E-cadherin protein (Ireton et al., 2002), and that it can regulate the turnover of cadherins in general (Davis et al., 2003; Xiao et al., 2003). To evaluate these observations in an embryonic context, we analyzed cadherin expression in embryos injected at the 1-cell stage with p120 MO. Immunoblot analysis of embryonic lysates isolated at different developmental stages revealed that p120 levels were moderately reduced at early gastrula stage (stage 10) and considerably at neurula (stage 15) and the tailbud (stage 22) stages [Fig. 4A, see also Table S1 in Supplemental data (http://jcs.biologists.org/cgi/content/full/117/18/4325/DC1)]. We noticed that depletion of p120 decreased the expression levels of C-
cadherin considerably, while E-cadherin protein was moderately reduced at the highest concentration of p120 MO and only at early gastrula stage. Interestingly, the levels of N-cadherin were not affected (Fig. 4A). The co-injection of p120 MO also strongly decreased the expression level of ectopic mouse E-cadherin, but not Myc-tagged *Xenopus* N-cadherin in *Xenopus* embryos (Fig. 4B). Because the cadherins are injected as synthetic RNA, the suppression of cadherin expression by p120 depletion must occur at the post-transcriptional level. The more potent effect of p120 depletion on ectopic versus endogenous E-cadherin suggests that p120 levels may be more rate limiting for ectopic (and overexpressed) cadherin than for endogenous cadherin protein.

The decrease in C- and E-cadherin protein induced by p120 MO was clearly not sufficient to induce any effect in a blastomere adhesion assay (data not shown). Neither did it affect the re-integration of transplanted aggregates in the blastocoel roof assay (data not shown). Both of these assays have been used before to demonstrate modulations in cadherin-dependent adhesion (Brieher and Gumbiner, 1994; Wacker et al., 2000). Taken together, p120 depletion appears to affect the expression levels of endogenous or ectopic E- and C-cadherin, while the levels of N-cadherin are not affected.

Expression of p120-uncoupled or JMD-deleted E-cadherin mutants also affects the eyes and the craniofacial skeleton

The highly conserved JMD of classical cadherins is crucial for the modulation of N-cadherin-mediated cell-cell adhesion during *Xenopus* embryogenesis (Kintner, 1992). As p120 interacts with the JMD, it can be considered the major candidate molecule for regulating the function of cadherins. A minimal core region within the JMD of E-cadherin is responsible for binding to p120 (Thoreson et al., 2000). To address the importance of p120 binding to the functionality of cadherins during early embryonic development, we generated two p120-uncoupled *Xenopus* E-cadherin variants, XE-cadh750AAA and XE-cadh753AAA, by mutating either the conserved amino acid residues 750-753 (GGG) or 753-756 (EED) of the XE-cadherin JMD to a triplet of alanines (AAA). The corresponding mutations in human E-cadherin disrupt the interaction with p120 either completely (by hE-cadh764AAA) or to a large extent (by hE-cadh761AAA) (Thoreson et al., 2000). Dorsal-animal overexpression of these two E-cadherin mutants induced defects in the eyes and the craniofacial skeleton similar to those observed upon injection of p120 MO (Fig. 5A,B and Table 3). However, the reduction of the eyes in embryos injected with the p120-uncoupled point mutants

<table>
<thead>
<tr>
<th>% Normal</th>
<th>% Mild eye phenotype*</th>
<th>% Severe eye phenotype*</th>
<th>Total scored (n)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control MO (8.33 ng-16.65 ng)</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>p120 MO (8.33 ng-16.65 ng)</td>
<td>4</td>
<td>16</td>
<td>80</td>
<td>103</td>
</tr>
<tr>
<td>+10 pg p120iso1</td>
<td>3</td>
<td>62</td>
<td>35</td>
<td>31</td>
</tr>
<tr>
<td>+20 pg p120iso1</td>
<td>0</td>
<td>77</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>+30 pg p120iso1</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td>48</td>
</tr>
<tr>
<td>+33 pg DN-Rac</td>
<td>42</td>
<td>46</td>
<td>12</td>
<td>56</td>
</tr>
<tr>
<td>+33 pg CA-Rac</td>
<td>5</td>
<td>28</td>
<td>67</td>
<td>20</td>
</tr>
<tr>
<td>+125 pg WT-RhoA</td>
<td>26.5</td>
<td>26.5</td>
<td>47</td>
<td>32</td>
</tr>
<tr>
<td>+45 pg DN-RhoA</td>
<td>8</td>
<td>27</td>
<td>65</td>
<td>26</td>
</tr>
<tr>
<td>+500 pg DN-LIMK</td>
<td>24</td>
<td>74</td>
<td>2</td>
<td>59</td>
</tr>
<tr>
<td>+150 pg CA-ROCK</td>
<td>15.5</td>
<td>61</td>
<td>23.5</td>
<td>26</td>
</tr>
</tbody>
</table>

*Mild eye phenotype, one or two eyes are substantially smaller; severe eye phenotype, one eye is absent or only rudiments are visible.*
Expression of p120-uncoupled E-cadherin affects the size of the eyes and cartilage. (A) Embryos were injected at the 8-cell stage in the dorsal-animal region and scored at stage 42 for the eye phenotype (a,c) and stage 46 for the cartilage phenotype after Alcian Blue staining (b,d). Tadpoles injected with 2 ng XE-cadh753AAA showing one smaller eye (c,d) compared with control injected embryos (a,b). (B) Graph representing the effect of XE-cadh753AAA RNA on the size of the craniofacial cartilage. Three normalized experiments are represented in one graph. The size of the head was calculated as in the legend of Fig. 3. The effect of XE-cadh753AAA can be partially rescued by co-injection of 150 pg wild type (WT) RhoA or 50 pg dominant negative (DN) Rac (values are statistically different from those injected with XE-cadh753AAA RNA alone).

Table 3. Percentages of eye phenotype scored after RNA injection

<table>
<thead>
<tr>
<th>Injected RNA</th>
<th>% Normal</th>
<th>% Eye phenotype</th>
<th>Total scored (n)</th>
<th>No. of experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-gal (2 ng)</td>
<td>99</td>
<td>1</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>XE-cadh750AAA (2 ng)</td>
<td>73</td>
<td>27</td>
<td>70</td>
<td>2</td>
</tr>
<tr>
<td>XE-cadh753AAA (2 ng)</td>
<td>35.5</td>
<td>64.5</td>
<td>101</td>
<td>6</td>
</tr>
<tr>
<td>+150 pg WT-Rho</td>
<td>83.5</td>
<td>16.5</td>
<td>95</td>
<td>6</td>
</tr>
<tr>
<td>+50 pg DN-Rac</td>
<td>88</td>
<td>12</td>
<td>91</td>
<td>5</td>
</tr>
<tr>
<td>+500 pg DN-LIMK</td>
<td>73.5</td>
<td>26.5</td>
<td>67</td>
<td>2</td>
</tr>
<tr>
<td>DN-RhoA (50 pg)</td>
<td>25</td>
<td>75</td>
<td>91</td>
<td>4</td>
</tr>
<tr>
<td>WT-RhoA (150 pg)</td>
<td>98</td>
<td>2</td>
<td>88</td>
<td>4</td>
</tr>
<tr>
<td>CA-RhoA (50 pg)</td>
<td>96</td>
<td>4</td>
<td>87</td>
<td>4</td>
</tr>
<tr>
<td>DN-Rac (50 pg)</td>
<td>97</td>
<td>3</td>
<td>68</td>
<td>3</td>
</tr>
<tr>
<td>CA-Rac (50 pg)</td>
<td>39</td>
<td>61</td>
<td>64</td>
<td>3</td>
</tr>
<tr>
<td>DN-LIMK (500 pg)</td>
<td>83</td>
<td>17</td>
<td>42</td>
<td>2</td>
</tr>
</tbody>
</table>
Expression of JMD-deleted E-cadherin affects the eyes and cartilage. (A) Embryos were injected at the 8-cell stage in the dorsal-animal region and scored at stage 42 for the eye phenotype (a,c) and stage 46 for the cartilage phenotype after Alcian Blue staining (b,d). Embryos injected with 1 ng mE-cadhΔJMD show malformation of the eye (c,d) compared with control injected embryos (a,b). The arrowhead in (b) indicates a patch of retinal pigmentation in the brain. (B) Graph representing the effect of mE-cadhΔJMD on the size of the craniofacial cartilage. Three normalized experiments are represented in one graph. The width of the head was calculated as in the legend of Fig. 3. The effect of mE-cadhΔJMD can be partially rescued by co-injection of 150 pg wild type (WT) RhoA or 50 pg dominant negative (DN) Rac (values are statistically different from those injected with mE-cadhΔJMD RNA alone).

The craniofacial cartilage is derived from the cranial NCCs that emigrate from the dorsal hindbrain into the branchial arches (Helms and Schneider, 2003). The reduced width and thickness of the cartilage structures of embryos injected with p120 MO, or with the p120-uncoupled or JMD-deleted cadherins, can be explained by impaired migration of the neural crest from the neural tube into the branchial arches, or by a reduced proliferation and/or increased apoptosis rate of the cells after they have properly migrated to their final position. To evaluate the migratory behavior of the cranial NCCs directly in vivo, we co-injected p120 MO or mE-cadhΔJMD RNA with a photo-activatable form of Fluorescein coupled to dextran. At the early neurula stage, the Fluorescein was activated in the region where the forming cranial NCCs are located. The migration of these cells into the branchial arches was then followed in real time. While the three streams of cranial NCCs originating at the dorsal side of the neural tube were broad and extended to a very ventral position in 90% of the embryos injected with the control MO (59 scored in four experiments), these streams were clearly aberrant or reduced in 64% of the embryos injected with p120 MO (35 scored in four experiments) or in 86% of the embryos injected with mE-cadhΔJMD RNA (38 scored in three experiments) (Fig. 9A). Interestingly, histological analysis of mE-cadhΔJMD-injected embryos (which have the strongest cartilage phenotype) showed large clumps of cells at stage 26 that were either associated with, or in close proximity to, the neural tube (Fig. 9B). This suggests that either the NCCs are unable to delaminate from the neural tube or they are unable to disperse after delamination.
In summary, the experiments with p120 MO and mE-cadhΔJMD RNA indicate that p120 and the JMD of cadherins are essential for the early stages of morphogenesis in the anterior neural tube, including the evagination of the eye vesicles, and for the proper migration of cranial NCCs into the branchial arches.

**Dominant negative (DN) Rac1 and wild-type (WT) RhoA rescue the eye and craniofacial phenotypes**

As it has been reported in the literature that expression of p120 in cell lines inhibits RhoA and activates Rac, we wondered whether a possible link exists between the observed eye and craniofacial malformations and the regulation of RhoA and Rac GTPases. Thus, we performed rescue experiments by injecting Xp120 MO, synthetic mRNA encoding p120-uncoupled, or JMD-deleted E-cadherin mutants, in combination with either wild type or mutant forms of RhoA and Rac1. Strikingly, coexpression of dominant negative (DN) Rac rescued all the eye and craniofacial phenotypes, whether induced by depletion of p120 (Fig. 3B, Tables 2, 3) or by expression of p120-uncoupled and JMD-deleted E-cadherin mutants (Fig. 5B, Fig. 6B, Table 3 and data not shown). Wild-type (WT) RhoA always had a less-effective rescuing activity than DN Rac and did not significantly rescue the craniofacial malformations induced by p120 MO and the eye phenotype of the JMD-deleted E-cadherin mutant. Interestingly, single injection of DN RhoA or constitutively active (CA) Rac1 strongly affected the anterior structures of the embryos, most notably the eyes (Table 3) and the craniofacial skeleton (data not shown).

To investigate the involvement of Rho GTPase activity in the observed phenotypes, we analyzed whether wild type or mutant versions of LIM kinase (LIMK) and Rho kinase (ROCK), known downstream effectors of the Rho GTPases that are described to be regulators of the actin cytoskeleton, would be able to rescue the phenotypes induced by p120 depletion and by ectopic expression ofXE-cadh753AAA or mE-cadhΔJMD (Tables 2, 3 and data not shown). We found that a constitutively active form of ROCK seriously affected the gastrulation movements. DN ROCK, wild type (WT) and DN LIMK had no major effect on development. Interestingly, DN LIMK could rescue the eye and craniofacial phenotypes induced by p120 MO (Table 2 and Fig. 3B), XE-cadh753AAA and mE-cadhΔJMD RNA (Table 3 and data not shown). Consistent with the results obtained with WT RhoA, we noticed that CA ROCK was less potent in rescuing the eye and craniofacial phenotypes induced by p120 MO (Table 2, Fig. 3B and data not shown).

**Dominant negative (DN) Rac1 and wild-type (WT) RhoA affect the localization and activity of cadherins in the embryo**

To examine how the Rho GTPases execute their rescue, we analyzed their effect on cadherin expression and localization in migrating cranial NCCs. The lack of proper antibodies to evaluate endogenous cadherins involved in the morphogenetic phenotypes prompted us to evaluate the effects of the Rho GTPases on the ectopic mE-cadherin, for which good antibodies for immunohistochemistry are available. We co-injected mE-cadhΔJMD, which on its own generates severe eye and craniofacial perturbations, with rescuing concentrations of WT...
RhoA and DN Rac, and analyzed ectopic cadherin expression and localization in the migrating cranial NCCs. Whereas mE-cadhΔJMD is strongly expressed and evenly distributed in NCCs when injected alone (Fig. 10A, panels a and d), its localization was more scattered in NCCs coexpressing DN Rac (Fig. 10A, panels b,e), and mE-cadhΔJMD protein levels were reduced when WT RhoA was coexpressed (Fig. 10A, panels c,f). This suggests that DN Rac, and to a lesser extent WT RhoA, can reduce the intercellular adhesiveness between the cranial NCCs, and hence facilitate the emigration of this cell population from the neural tube and its migration into the branchial arches.
p120 catenin in vertebrate morphogenesis 4335

plasmid is unevenly distributed between the cells with successive cell divisions, the injected plasmid generates mosaic expression of the EGFP fusion protein in the developing embryos. Consequently, we noticed various expression levels of αE-catenin-EGFP. Because of the limited transparency of the Xenopus embryos at the earlier developmental stages, we could only evaluate the localization of the fusion protein in the outer cell layers and not in the NCCs. We noticed that αE-catenin-EGFP is clearly localized at cell-cell contacts in control-injected embryos (Fig. 10B, panel a). Strikingly, when mE-cadhΔJMD was co-injected, strong enrichment of αE-catenin-EGFP proteins was visible at cell-cell contacts (Fig. 10B, panel b). The intercellular contacts mostly formed straight lines, suggesting that cortical tension is strong and cells are robustly associated. We also noticed a higher tendency for cells coexpressing the αE-catenin-EGFP and mE-cadhΔJMD to be grouped together, while they were more dispersed in control injected embryos. This suggests that cells derived from the same precursor stay more associated in embryos injected with mE-cadhΔJMD than in control-injected embryos. When DN Rac was co-injected with mE-cadhΔJMD, the concentration of αE-catenin-EGFP was greatly reduced (Fig. 10B, panel c), and the evaluation of different Z-sections revealed that its localization at cell-cell contacts was limited to a fine line (data not shown). Conversely, the co-injection of WT RhoA had no effect on the localization of αE-catenin-EGFP.

Taken together, the microscopic analysis of sections and the in vivo experiments suggest that DN Rac affects the concentration of cadherin at the membrane, and has a strong influence on the interaction of cadherins with α-catenin. Whereas, WT RhoA primarily seems to change the expression and/or function of the cadherin, while the association with α-catenin is not affected.

Discussion

Studies in cell lines indicate that p120 is ideally positioned to transfer signals from the cadherins to the cytoskeleton (outside-in signaling), and to transmit intracellular signaling events that influence the activity and/or expression levels of the cadherin at the membrane (inside-out signaling). While studies in C. elegans and Drosophila have indicated that p120 is dispensable for development in invertebrates, it was shown recently that p120 is an essential factor in vertebrate development, and is required for normal gastrulation and convergent extension movements (Fang et al., 2004). In our study we documented an essential role for p120 in the morphogenetic movements associated with the formation of the eyes and the craniofacial skeleton and analyzed the molecular mechanism by which p120 exerts this control.

Our data indicate that p120 is involved in regulating morphogenesis in early Xenopus embryos, especially in derivatives of the ectodermal cell lineage. Firstly, we demonstrate that while basal levels of p120 transcripts are expressed throughout the embryo, mRNA expression is particularly enriched in embryonic regions that undergo extensive morphogenetic movements. This is especially true for tissues, such as the head (neuro)ectoderm, the eye vesicles and the cranial neural crest, a highly migratory cell population that originates at the border of the anterior neural plate and the future epidermis. Secondly, we show that morpholino (MO)-
mediated depletion of p120 affects the formation of the eyes and the craniofacial skeleton, structures that are derived from tissues in which p120 messenger is highly enriched, i.e. the eye vesicle and the cranial neural crest. Thirdly, using in vivo labeling techniques, we show that the evagination of the eye vesicles from the brain is disturbed in p120-depleted embryos, as is the migration of the cranial NCCs from the dorsal side of the neural tube into the branchial arches.

We found that depletion of p120 decreased the endogenous or ectopic levels of C- and E-cadherin proteins, which is consistent with recent reports describing similar effects of p120 in cell culture (Davis et al., 2003; Xiao et al., 2003) and in Xenopus embryos (Fang et al., 2004). However, N-cadherin levels were not affected. This may correlate with the presence of one or two of the tyrosine residues suspected in Hakai-mediated degradation in C- and E-cadherin, respectively, and their absence in N-cadherin (Fujita et al., 2002). Presently, however, it is not clear whether the reduced C- and E-cadherin levels are related to the observed malformations in the eyes and the craniofacial skeleton, whose progenitors presumably do not even express C- or E-cadherin.

Interestingly, we could phenocopy the eye and craniofacial malformations by overexpression of XE-cadherin derivatives that were point mutated in the p120 binding site. Similar to p120 depletion, these cadherin mutants, which no longer or only weakly bind p120, reproducibly affected eye development, albeit less prominently than p120 MO. They also reduced the width of the head. We found that these mutant cadherins are poorly expressed, which may explain their weaker phenotype. Dorso-animal expression of a mE-cadherin derivative that contains a 31 amino acid deletion in the JMD, covering both the p120 and the Hakai-binding sites, affected the formation of the eyes and the craniofacial cartilage more severely. However, in this case the eye phenotype was clearly different from that obtained after p120 depletion or expression of a p120-uncoupled point mutated E-cadherin. Rather than being reduced in size or totally absent, the eyes were very irregularly shaped, and retinal pigmentation could often be observed in the optic stalk and in patches of cells that were located within the brain. Because the JMD-deleted E-cadherin mutant is more strongly localized at cell-cell contacts compared with full length E-cadherin, the phenotypes could be explained by the inability of some cells to turn down cadherin expression or functionality. Evidently, because a 31 amino acid region is missing from this mutant, binding to components other than p120 and Hakai may be disrupted. This may prevent dynamic regulation of this cadherin, which is necessary for the correct morphogenetic movements associated with eye and jaw formation. The malformations of the craniofacial skeleton were most severe for this deletion mutant. In addition to being smaller, the lower jaws had malformations severe enough to prevent closing of the mouth. Microscopic inspection of the alcian-blue-stained craniofacial cartilage skeleton indicated a greatly reduced number of cells.
in these structures in embryos injected with p120 MO or with the JMD-deleted E-cadherin.

In vivo labeling of specific cell populations in embryos injected with p120 MO or JMD-deleted E-cadherin indicated that both the correct evagination and/or proximal constriction of the eye vesicle were affected, and the migration of the cranial NCCs was reduced. This indicates that the small eyes and the reduced craniofacial cartilage structures are primarily the result of impaired morphogenetic behavior, and not of defects in cell proliferation and/or specification. We also observed that p120 MO, and to a lesser extent JMD-deleted E-cadherin, affected the structure of the anterior neural folds, which caused aberrant closure of the anterior neural plate. It is possible that apical cellular constrictions, which are probably needed to allow the correct formation of the neural ridges and the infolding of the anterior neural tube, are disturbed by p120 depletion. Importantly, we observed that upon injection of p120 MO or the p120-uncoupled E-cadherin point mutants, the eyes were always well separated from the brain, even when they were considerably reduced in size. Actually, we never observed cyclopia, which normally results from incomplete separation of the presumptive eye field in the anterior neural plate, and can be a sign of general ventralization of the embryo and/or deficient signaling from the prechordal plate that moves underneath the eye field and induces its separation. This indicates that the reduced size of the eyes does not result from changes in the anterior-posterior patterning of the embryo.

The impaired migration of the cranial NCCs depleted of p120 or expressing the JMD-deleted E-cadherin mutant raises the important question of why these cells are curtailed in their migratory behavior. This could be the result of impaired cell-cell adhesion, a failure of the NCCs to respond to the migratory cues in the tissues they have to transverse, or in the light of our results with the Rho GTPases (see next paragraph), from a cytoskeletal condition unsupportive of migration, or from a combination of any these processes. The JMD-deleted E-cadherin induced the strongest phenotype in the cranial cartilage, and histological inspection of the head region of embryos injected with the JMD-deleted E-cadherin mutant indicated the presence of tightly packed cells flanking the neural tube. These are probably NCCs that have delaminated but failed to disperse. Immunolabeling of cells expressing the JMD-deleted cadherin showed that these cells are tightly packed, and display continuously high concentrations of the cadherin at the intercellular contacts. This suggests, at least for cranial NCCs expressing the JMD-deleted E-cadherin, that intercellular adhesion is too robust or static to permit the efficient emigration of these cells from the dorsal neural tube region into the branchial arches. Our results also imply that NCCs, aside from changing cadherin patterns (Nakagawa and Takeichi, 1998; Pia et al., 2001), also need to suppress cadherin functionally to delaminate and migrate. This is in agreement with our observation that overexpressed wild-type mE-cadherin does not impair NCC migration, and actually competitively rescues the phenotype generated by the JMD-deleted E-cadherin. This is further in line with results in avian embryos, where it was shown that in migrating NCCs, N-cadherin is expressed, but is not located at the cell surface and is not connected to the cytoskeleton (Monier-Gavelle and Duband, 1995). Interestingly, inhibitors of protein kinases (including tyrosine kinases) restored N-cadherin-mediated cellular contacts and prevented migration. These results imply that NCCs possess a mechanism for suppressing N-cadherin function. The same mechanism may be responsible for the functional suppression of ectopically expressed wild-type E-cadherin in our experimental set-up, or it may regulate the activity of endogenous Xcadherin-11, which is the major cadherin expressed in the cranial NCCs of Xenopus and is involved in its migration and specification (Borchers et al., 2001).

Intriguing is the ability of dominant negative (DN) Rac, and to a lesser extent wild type (WT) RhoA, to rescue the phenotypes induced by p120 depletion, or by overexpression of the p120-uncoupled and JMD-deleted cadherin mutants. This implies that manipulation of Rho GTPase activity can relieve the restrictions imposed by these treatments. In line with these results, we found that DN RhoA and CA Rac also induce smaller eyes and heads. Because Rho GTPases have been implicated in various biological processes, including regulation of actin dynamics, cell adhesion, transcription, cell growth and membrane trafficking (reviewed by Etienne-Manneville and Hall, 2002), we can envision different scenarios by which Rho GTPases affect morphogenetic movements. However, several reports support a direct role for the Rho GTPases in cadherin-mediated cell adhesion, with the strongest case for the involvement of Rac (Fukata and Kaibuchi, 2001). Moreover, p120 has been shown to regulate cell motility through regulation of the Rho GTPases (Anastasiadis et al., 2000; Grosheva et al., 2001; Noren et al., 2000). The observation that DN LIMK, a downstream effector of Rac that plays a role in the regulation of the actin cytoskeleton, is also efficient in rescuing the eye and craniofacial phenotypes, hints that changes in the actin cytoskeleton are associated with the observed morphogenetic phenotypes. More experiments are needed to clarify the involvement of the Rho GTPases at the mechanistic level.

Our results do not allow us to draw unambiguous conclusions on how p120 depletion affects morphogenetic behavior in general and cadherin function in particular. Because part of the phenotypes of p120 depletion can be phenocopied by the expression of the p120-uncoupled and JMD-deleted cadherin mutants, and as the latter clearly increases intercellular contacts in the embryonic context, it is tempting to speculate that p120 depletion also reinforces cadherin-mediated adhesion in this experimental setting. This would also clarify the comparable rescuing activities of DN Rac, DN LIMK, WT RhoA and CA ROCK. Unfortunately, we currently lack the appropriate tools for investigating this scenario. In conflict with such a conclusion are, of course, recent reports that clearly show that p120 stabilizes cadherin expression, at least in cell lines (Davis et al., 2003; Xiao et al., 2003). It is, however, quite possible that this activity is dependent on the cellular context, and it has often been found that cells in a natural three-dimensional context acquire properties different from those developed when growing on an artificial substrate. In accordance with this, we clearly showed that in our experimental setup N-cadherin expression is not affected by p120 depletion. Hence, cell-cell adhesion exerted by N-cadherin or another cadherin (e.g., cadherin-11), may not be functionally suppressed in the morphogenetic tissues upon p120 depletion. Interestingly, mis-expression studies in Xenopus have shown that NCCs overexpressing either full...
length cadherin-11, the major cadherin expressed in migrating NCCs in *Xenopus*, or a DN mutant lacking the β-catenin binding site, fail to migrate efficiently into the branchial arches (Borchers et al., 2001). This implies that both too much and too little adhesion affect the migration of these cells. Evidently, further research is needed to discover exactly how p120 depletion influences the different morphogenetic processes.

In conclusion, our work demonstrates that in contrast to invertebrates, p120 is essential for correct morphogenesis in vertebrate embryos. By acting directly on the activity of the cytoskeleton and the vertebrate embryos, p120 is essential for correct morphogenesis in vitro. Depletion influences the different morphogenetic processes. Too little adhesion affect the migration of these cells. Evidently, binding site, fail to migrate efficiently into the branchial arches. Evidently, binding site, fail to migrate efficiently into the branchial arches.

We would like to thank H. Funakoshi, T. Nakamura, K. Kaibuchi, B. Janssens, A. Hall, M. Takeichi, B. Gumbiner, M. Ozawa and R. Kemler for their generous gifts of plasmdids and antibodies. We are indebted to E. Parthoens for excellent support with confocal microscopy, to P. De Bleser for help with bioinformatics, to A. Servesa for aid throughout the project and to A. Cauweels and A. Bredan for critical reading and editorial assistance. Research is supported by grants from the Belgian Federation against Cancer and the Interuniversitaire Attractiepelen. M.D. and V.V. are fellows with the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Flanders). K.V. is a post-doctoral fellow with the FWO-Vlaanderen.

References


