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Dynamic Expression Patterns of RhoV/Chp and RhoU/Wrch During Chicken Embryonic Development

Cécile Notarnicola,1 Ludovic Le Guen,1 Philippe Fort,2 Sandrine Faure,2† and Pascal de Santa Barbara1*†

Rho GTPases play central roles in the control of cell adhesion and migration, cell cycle progression, growth, and differentiation. However, although most of our knowledge of Rho GTPase function comes from the study of the three classic Rho GTPases RhoA, Rac1, and Cdc42, recent studies have begun to explore the expression, regulation, and function of some of the lesser-known members of the Rho GTPase family. In the present study, we cloned the avian orthologues of RhoV (or Chp for Cdc42 homologous protein) and RhoU (or Wrch-1 for Wnt-regulated Cdc42 homolog-1) and examined their expression patterns by in situ hybridization analysis both during early chick embryogenesis and later on, during gastrointestinal tract development. Our data show that both GTPases are detected in the primitive streak, the somites, the neural crest cells, and the gastrointestinal tract with distinct territories and/or temporal expression windows. Although both proteins are 90% identical, our results indicate that cRhoV and cRhoU are distinctly expressed during chicken embryonic development.

Key words: Rho GTPases; RhoV; RhoU; neural crest; gastrointestinal tract development; chick embryo

INTRODUCTION

Rho family GTPases are crucial regulators of a variety of cellular functions, including actin cytoskeleton dynamics, cell adhesion and motility, vesicular trafficking, cell growth, and survival (Etienne-Manneville and Hall, 2002; Wennerberg and Der, 2004; Hall, 2005). Like most other Ras-like members, Rho GTPases oscillate between inactive GDP-bound and active GTP-bound states, the switch of their activity being tightly regulated by the auxiliary proteins GEFs, GAPs, and GDIs (Van Aelst and D’Souza-Schorey, 1997; Rossman et al., 2005). Once activated, Rho GTPases interact with specific downstream effectors and regulate a variety of intracellular processes including the F-actin cytoskeleton dynamics, gene transcription, and activation of kinase cascades, such as the JNK and p38 MAP kinases (Fort, 1999; Ridley, 2001; Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2002). Although the Rho family of GTPases is made of 20 members in vertebrates, little is known on the cellular and physiological roles of most Rho members, because studies published to date have focused on RhoA, Rac1, and Cdc42, the most conserved and expressed members from lower eukaryotes to mammals (Boureux et al., 2007).

Among the Rho members found early in eukaryotes, RhoV (also designed as Chp for Cdc42 homologous protein; Aronheim et al., 1998) and its close relative RhoU (also designed as Wrch-1 for Wnt-1–regulated Cdc42 homolog; Tao et al., 1998) form a sep-
arate branch of the Rac/Cdc42 sub-

family with atypical features (Boureux et al., 2007; Aspenström et al., 2007). Indeed, although RhoV and RhoU share functional similarities with Rac- and Cdc42-related proteins, they both possess additional N- and C-terminal sequences that distinguish them from the classic Rho structure (Shutes et al., 2006). In addition, they both lack the canonical C-terminal CAAX motif required for prenylation and require palmitoylation for membrane association, suggesting that they might exert their activities in distinct subcellular locations compared with other Rho members (Berzat et al., 2005; Chenette et al., 2006; Shutes et al., 2006). The high similarities in protein sequence and biochemical properties between RhoU and RhoV suggest that the two proteins might fulfill redundant biological functions. Along this line, both proteins exhibit intrinsic transforming activities, whereas other Rho members only cooperate in cell transformation (Berzat et al., 2005; Chenette et al., 2005, 2006). We also recently showed that RhoU can rescue the loss of RhoV function during neural crest development in *Xenopus* (Guémar et al., 2007). One could imagine that the specific role of RhoV and RhoU depends on their expression pattern.

In the present study, we examined the expression profiles of RhoV and RhoU from Hamburger and Hamilton (HH) stages 5 to 12 of chick development, and later on, during gastrointestinal tract development. In the light of their expression patterns, we discuss the relationships between both GTPases in terms of developmental functions.

RESULTS AND DISCUSSION

Cloning and Sequence Analysis of cDNAs Encoding Chick RhoV and RhoU

To study the expression patterns of RhoV and its closest relative RhoU during chicken embryonic development, we first searched chick cDNA sequences coding for rat RhoV (accession no. AF0978871.1) and human RhoU (accession no. AF378087.1) orthologues using the NCBI tblastn program (http://www.ncbi.nlm.nih.gov/blast). The search identified several putative chicken mRNA sequences (XM_426425.2 for RhoV, XM_426146 and XM_427270 for RhoU) predicted by automated computational analysis (Gnomon, NCBI). RhoV XM_426425.2 was derived from a three-exon gene structure (ENSGALG00000008476, http://www.ensembl.org), in consistency with RhoV and RhoU gene structures in vertebrates (Boureux et al., 2007), and encodes a full-length protein that shares 82% amino acid (aa) identity with rat RhoV. The two putative RhoU transcripts were derived from the partially assembled ENSGALG00000008476, http://www.ensembl.org), in consistency with RhoV and RhoU gene structures in vertebrates (Boureux et al., 2007), and encodes a full-length protein that shares 82% amino acid (aa) identity with rat RhoV. The two putative RhoU transcripts were derived from the partially assembled ENSGALG00000011073 locus: XM_426146, spanning over the putative second and third exons and encoding aa 89-228 (compared with human RhoU) and XM_427270, spanning over the last 108 N-terminal (nt) of the second intron and over the third exon (456 nt coding for aa 107-258, as well as 2.5 kb of 3’ noncoding region). Searches in the chick expressed sequence tag (EST) database produced no EST for RhoV and 20 for RhoU, all located in the 3’ noncoding region except BU477343 and BU255458, encoding the 41 and 7 Carboxyterminal amino acids, respectively. Such long putative 3’ noncoding region is in agreement with those observed in human (NM_021205, 2.9 kb) or mouse (NM_133955, 2.5 kb). Neither in EST nor in genomic databases could we detect the RhoU N-terminal sequence (i.e., homologous to the 88 amino acids in the human first exon). The partial RhoU protein (aa 89-258) shares 98% identity with human RhoU. Protein sequence alignments and phylogenetic analyses confirmed that the identified chick genes are bona fide RhoV and RhoU orthologues that we will refer to as cRhoV and cRhoU (Fig. 1). We next generated in situ hybridization probes by reverse transcriptase-polymerase chain reaction (RT-PCR) and cloning of RhoV and RhoU open reading frame (ORF) fragments from HH stage 8 embryo mRNA (see the Experimental Procedures section).

**cRhoV and cRhoU Expression Patterns During Early Development**

We next examined the spatiotemporal expression patterns of cRhoV and cRhoU during early development by whole-mount in situ hybridization of embryos from HH stages 5 to 12 (Hamburger and Hamilton, 1951). At HH stage 5, both cRhoV and cRhoU...
are expressed throughout the primitive streak and in Hensen's node (Figs. 2A, 3A). In addition, cRhoU expression, detected in the entire epiblast, is excluded from the most anterior region of the embryo (black arrowhead in Fig. 3A) but is enriched in an elliptical region centered over the Hensen's node corresponding to the prospective anterior neural plate. As development proceeds, expression of cRhoU decreases in the primitive streak and in the adjacent posterior open neural plate (Fig. 3B,C), while it is reinforced both in the head fold and in mesodermal cells lateral to the open neural plate as indicated by transverse sections (Fig. 3C,C'). From HH stage 7, expression of cRhoV is reinforced posteriorly in the primitive streak as shown by transverse sections (Fig. 2B,B'), while cRhoU is no longer detected in this structure from HH stage 7 (Fig. 3C,C'). At HH stage 8, both cRhoV and cRhoU are expressed in the head neural folds (Figs. 2C,D, 3D,E), although highest levels of cRhoV transcripts are detected in the primitive streak (Fig. 2C,C'). At this stage, both GTPases are also expressed in the mesoderm of the GI tract, while undetectable in the unsegmented somite mesoderm (Figs. 2C,D, 3D,E). At later developmental stages, stronger cRhoV expression is detected in the anterior neural folds and also at weakest levels from HH stage 9 in the posterior neural folds as well (Fig. 2E–I). Surprisingly, examination of transverse sections reveals disjunctive cRhoV and cRhoU patterns in the anterior neural folds, cRhoV transcripts being predominantly found in the ectoderm adjacent to the neural folds but absent from the neural folds and the neural plate (Fig. 2F,F',G,G'), while cRhoU transcripts are restricted to the dorsal-most region of the neural tube and undetectable from the surrounding ectoderm (Fig. 3E,E'). Of interest, cRhoV expression profile in the neural folds resembles that of the neural crest inducer Wnt6 (Garcia-Castro et al., 2002), while the cRhoU pattern in the dorsal part of the neural tube is similar to Bmp4 (Garcia-Castro et al., 2002). These observations in the chick embryo are consistent with our previous study identifying RhoV as an essential regulator of neural crest induction in Xenopus (Guémar et al., 2007).

At HH stage 9, in addition to the dorsal neural tube, cRhoU is expressed in the lateral plate mesoderm and in the somites (Fig. 3F,G,G'). As development proceeds, the broad expression of cRhoU in the dorsal side of the neural tube decreases; however, a stronger expression was detected in the head, both anteriorly in the olfactory placodes and laterally to the neural tube, in the migrating cranial neural crest cells at HH stage 10 (Fig. 3H,I,I'). In addition, expression of cRhoU remains restricted to the most dorsal part of the mature somites from HH stage 12 (Fig. 3L,L'), while it is expressed in the dorsoventral part of the epithelial somites from HH stage 8 (Fig. 3G,G'). At this stage, cRhoU is also detected in the optic vesicles and in the splanchnic mesoderm that will contribute to the mesoderm of the gastrointestinal (GI) tract (Fig. 3L,L').

cRhoV and cRhoU Expression Patterns During GI Tract Development

It was previously shown that RhoU mRNA is highly expressed in the adult stomach (Kirikoshi and Katoh, 2002), and RhoV mRNA has been reported to be up-regulated in several primary gastric cancers (Katoh, 2002). RhoU expression was also shown to be down-regulated in the jejunum of Wt1/Wt1 mice (Daigo et al., 2004), which show a reduced number of interstitial Cajal cells, which act as a pacemaker for the contraction of the visceral smooth muscles in association with the enteric nervous system (Lecoin et al., 1996). For these reasons, we next addressed the spatiotemporal distribution of cRhoV and cRhoU during GI tract development. In the primitive uniform GI tract, cRhoV expression is first detected at E4 in the foregut and the caudal part of the hindgut (Fig. 4A). At E5, a stronger cRhoV signal is detected in the gizzard (muscular stomach) and the cloaca (Fig. 4B,E,F). As demonstrated by transverse sections (Fig. 4C,D), cRhoV transcripts are restricted to the endodermal layer of these structures. A fainter cRhoV signal was also detected in the endoderm of the proventriculus (glandular stomach) at E6 (Fig. 4E). The expression pattern of cRhoU during GI tract development appeared much more dynamic. Its expression is first detected at E4 in the anterior foregut that will generate the lung, as well as in the glandular stomach, the caecum bud, and the caudal hindgut (Fig. 4G). A weaker but significant cRhoU signal was also detected in the midgut. At E5, expression of cRhoU invades the whole GI tract with the exception of the colon (Fig. 4I). It is expressed at high levels in the lung, the glandular stomach, the caecum, and the cloaca and at low levels in the small intestine and the gizzard. In all these structures, cRhoU expression is restricted to the mesodermal layer of these tissues, as previously reported for Bmp4 (Goldstein et al., 2005). It is noteworthy that, in the cloaca, cRhoU transcripts are restricted to the mesodermal cell layer (Fig. 4J–M), while cRhoV transcripts are localized to the adjacent endodermal layer (Fig. 4C,D). At E5 and E6, cRhoU expression is observed in the distal domain of the stomach as a ring corresponding to the pyloric sphincter, a muscular structure separating the stomach from the duodenum (Fig. 4L,N). At E6 and E7, cRhoU transcripts accumulate in the lung, the glandular stomach, the caecum, and the cloaca (Fig. 4N,O).

In conclusion, although cRhoV and cRhoU display a few overlapping domains of expression, they are mostly expressed in distinct territories and/or temporal expression windows. For instance, although both expressed in the primitive streak from HH stage 5, only cRhoV remains expressed in this structure by HH stage 7. Similarly, although the two genes are expressed in the newly segmented somites, only cRhoU is found in the mature somites. Although cRhoV and cRhoU are expressed in the forming cranial neural crest cells, only cRhoU is found in the migrating ones. Later during development, cRhoU is exclusively expressed in the mesoderm of the GI tract, while cRhoV is detected in the stomach epithelium. The differential expression of RhoV and RhoU supports a scenario according to which both proteins fulfill similar functions in specific tissues or at different times. Indeed, duplicated genes have been shown to be retained though evolution by neofunctionalization.
tion, in which mutations on one gene leads to a protein with new properties, or by subfunctionalization, in which the two genes become expressed in distinct territories through the modification of their regulatory elements (for a review, see Li et al., 2005). These mechanisms are not mutually exclusive, because once differentially expressed, each paralog has the capacity to evolve independently. The Rho family of GTPases illustrates both types of evolution: on the one hand, RhoA, Rac1, and Cdc42 perfectly match the neofunctionalization crite-

Fig. 2.

Fig. 3.
tion because they are coexpressed in many cell types from yeast to mammals and have gained specific properties in terms of subcellular distribution and binding partners (Bustelo et al., 2007). On the other hand, more recently duplicated genes such as Rac1-3 or RhoA-C proteins show distinct tissue distribution (Boureux et al., 2007), while exhibiting very similar biochemical properties, which clearly meets the subfunctionalization criterion. The present study strongly suggests that it is also the case for cRhoV and cRhoU, which predicts that the corresponding proteins still fulfill very similar cellular functions.

**EXPERIMENTAL PROCEDURES**

**Chick Embryos**

Timed fertilized white Leghorn eggs (Haas Farm, France) were incubated at 38°C in a humidified incubator (Coudelou, France) until used experimentally. Embryos were staged according to Hamburger and Hamilton (Hamburger and Hamilton, 1951) for early embryogenesis stages and by embryonic day (E) for gastrointestinal tract analysis. Whole embryos or dissected gastrointestinal tissues were fixed in 4% paraformaldehyde for 2 hr at room temperature, washed in phosphate buffered saline (PBS), gradually dehydrated in methanol to store the sample at −20°C before processing as described below.

**Cloning of Chick RhoV and RhoU**

Searches were performed in *Gallus gallus* gene and EST databases with the NCBH blastn program (http://www.ncbi.nlm.nih.gov/BLAST) using the *Rattus norvegicus* RhoV and the *Homo sapiens* RhoU proteins as queries. ORF fragments were obtained by RT-PCR from HH stage 8 chick embryos using the following primer sets: RhoV, forward 5′-ccg gct agt atg cc-3′, reverse 5′-cct gag cac gac tgt gta ggc atc c-3′; RhoU, forward 5′-ccg gct agt atg cc-3′, reverse 5′-ctc gag ctc cag tgt gcc agg atc c-3′. RT-PCRs were performed with pfu polymerase (30 cycles, 94°C 1 min, 57°C 30 sec, 72°C 2 min). PCR products were 474 bp for RhoV and 438 bp for RhoU, containing aa 1-158 and aa 89-234 of the corresponding human sequences, respectively. Fragments were subcloned into the PCR4-TOPO vector (Invitrogen) and checked on an ABI310 automatic sequencer (Perkin-Elmer, Foster city, CA) before subcloning in the pCS2+ vector.

**Whole-Mount In Situ Hybridization**

Whole-mount in situ hybridization was carried out as described by Faure et al. (2002) and Moniot et al. (2004). In summary, tissues were gradually rehydrated in PBS, washed in PBT (PBS, 0.1% Tween) and incubated for 1 hr in 6% hydrogen peroxide (Sigma, France). Samples were next permeabilized by treatment with proteinase K (10 μg/ml) for 5 min (early stage) and 10 min (gastrintestinal tract), washed with glycin in PBT and fixed in 4% paraformaldehyde/0.2% glutaraldehyde in PBT for 20 min. Tissues were then hybridized with anti-sense cRhoV and cRhoU digoxigenin-labeled (Roche) riboprobes overnight at 70°C. After posthybridization washes at 70°C, tissues were incubated in 10% sheep serum for 2.5 hr at room temperature and finally mixed with pre-absorbed anti-digoxigenin coupled

![Fig. 2. A–I: cRhoV expression pattern during early development. Hamburger and Hamilton (HH) stage 5 (A), stage 7 (B), stage 8 (C,D), stage 9 (E–G), and stage 10 (H,I) embryos were analyzed by whole-mount in situ hybridization using digoxigenin (DIG)-labeled RNA probes for cRhoV. Transverse sections were performed where indicated on HH stages 7, 8, and 9 embryos labeled (B,C,F,G). A: At HH stage 5, cRhoV is expressed in the primitive streak (arrow). B: At HH stage 7, cRhoV expression is detected in the posterior primitive streak and in the Hensen’s node. B′: Cross-section as indicated in B showing cRhoV expression in the primitive streak. C: At HH stage 8, cRhoV expression is detected in the head neural fold and in the remaining primitive streak. C′: Cross-section as indicated in C showing cRhoV transcripts localization in the primitive streak. D: Enlargement of C in the head fold level. E: At HH stage 9, cRhoV expression is observed in the anterior and posterior neural folds. F: Enlargement of E in the head fold level showing that cRhoV expression is detected in the head neural fold and in the epithelial somites. F′: Cross-section as indicated in F showing cRhoV expression in the ectoderm adjacent of the anterior neural fold. G: Enlargement of E in the posterior neural fold level showing cRhoV expression in the remaining primitive streak and in the posterior neural folds. G′: Cross-section as indicated in G showing cRhoV expression in the ectoderm adjacent to the posterior neural fold. H: At HH stage 10, cRhoV expression is mainly observed in the posterior primitive streak and in the neural folds. I: Enlargement of H at the head level showing cRhoV expression in the cranial neural folds. Ec, ectoderm; hn, Hensen’s node; nf, neural folds; np, neural plate; ps, primitive streak; so, somites.](image)

![Fig. 3. cRhoU expression pattern during early development. Hamburger and Hamilton (HH) stage 5 (A), stage 6 (B), stage 7 (C), stage 8 (D,E), and stage 9 (F,G), stage 10 (H,I), and stage 12 (J–L) embryos were analyzed by whole-mount in situ hybridization using digoxigenin (DIG)-labeled RNA probes for cRhoU. Transverse sections were performed where indicated on HH stages 7, 8, 9, 10, and 12 embryos labeled (C,E,G,H,I). A,B: cRhoU is expressed in the primitive streak, in the entire epiblast with the exception of the most rostral part of the embryo (arrowhead) at HH stages 5 (A) and 6 (B). C: At stage 7, cRhoU expression is reinforced in the head fold and lateral tissues. C′: Cross-section as indicated in C showing cRhoU expression in the lateral mesoderm. D: At HH stage 8, cRhoU expression is detected in the head neural fold, lateral tissues, and somites. E: Enlargement of D showing cRhoU expression in the undifferentiated somites. E′: Cross-section as indicated in E showing cRhoU transcripts localized in the dorsal-most region of the neural tube. F: At HH stage 9, cRhoU expression is observed in the dorsal neural tube and lateral mesoderm. G: Enlargement of F showing cRhoU expression in the dorsal neural tube and the somites. G′: Cross-section as indicated in G showing that cRhoU is expressed in the dorsoventral domains of the epithelial somites and in the lateral plate mesoderm. H: At HH stage 10, cRhoU expression is observed in the olfactory placodes, the cranial neural crest, the somite, and in the lateral mesoderm. I: Enlargement of H showing cRhoU expression in the cranial neural crest and the olfactory placodes. I′: Cross-section as indicated in I showing cRhoU expression in the migrating cranial neural crest. J: At HH stage 12, cRhoU expression is observed in the somites and lateral mesoderm. K: Enlargement of J showing cRhoU expression in the optic vesicles. L: Enlargement of J showing cRhoU expression in the mature somites and lateral mesoderm. L′: Cross-section as indicated in L showing that cRhoU is expressed in the dorsal mature somites and splanchnic lateral mesoderm. Ec, ectoderm; nf, head fold; hn, Hensen’s node; Ip, lateral plate mesoderm; mnc, migrating neural crest cells; nf, neural folds; op, olfactory placode; ov, optic vesicle; ps, primitive streak; so, somites; sp, splanchnic mesoderm.](image)
with alkaline phosphatase antibody (Roche) overnight at 4°C. The complexes were detected with BM purple solution (Roche). For sectioning, tissues were equilibrated in 30% sucrose in PBS, then embedded in OCT (Optimal Cutting Temperature Compound, Tissue-Tek) and sectioned at 20 μm on a cryostat.

**Photography**

Images were collected in whole-mount under a Nikon SMZ1000 scope and in section under a Zeiss Axiophot microscope, both using Nikon DXM1200 camera.

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