Global Inhibition of Lef1/Tcf-dependent Wnt Signaling at Its Nuclear End Point Abrogates Development in Transgenic Xenopus Embryos

Received for publication, August 5, 2004, and in revised form, September 13, 2004
Published, JBC Papers in Press, September 14, 2004, DOI 10.1074/jbc.M408969200

Tom Deroo§§, Tinneke Denayer§§, Frans Van Roy**, and Kris Vleminckx‡ ‡‡
From the §§Developmental Biology Unit and the ***Molecular Cell Biology Unit, Department for Molecular Biomedical Research, Ghent University-Flanders Interuniversity Institute for Biotechnology, 9052 Ghent, Belgium

Analysis of canonical Wnt signaling during vertebrate development by means of knock-out or transgenic approaches is often hampered by functional redundancy as well as pathway bifurcations downstream of the manipulated components. We report the design of an optimized chimera capable of blocking transcriptional activation of Lef1/Tcf-β-catenin target genes, thus enabling intervention with the canonical Wnt pathway at its nuclear end point. This construct was made hormone-inducible, both functionally and transcriptionally, and was transgenically integrated in Xenopus embryos. Down-regulation of target genes was clearly observed upon treatment of these embryos with dexamethasone. In addition, exposure of variously aged transgenic embryos to dexamethasone caused complex phenotypes with many new but also several recognizable features stemming from inhibition of canonical Wnt signaling. At least in some tissues, a significant reduction in cell proliferation and an increase in programmed cell death appeared to underlie these phenotypes. Our inducible transgenic system can serve a broad range of experimental settings designed to unveil new functional aspects of Lef1/Tcf-β-catenin signaling during vertebrate embryogenesis.

During animal embryogenesis, communication mediated by a miscellaneous cast of secreted signaling factors controls many of the complex interactions within cellular assemblies that are necessary for the correct patterning of tissues and the creation of functional organs. Secreted glycoproteins of the Wnt family take an eminent place among such signaling factors, and their widespread occurrence in the embryo opens the opportunity to identify transcriptional targets within the primate end of the macromolecule. Our inducible transgenic system serves as a powerful tool for the study of the transcriptional targets of Lef1/Tcf-β-catenin signaling.

Canonical Wnt signaling proceeds through an evolutionarily conserved pathway and starts at the cell membrane where a Wnt ligand stimulates a Frizzled-LRP5/6 receptor complex (5, 6). Intracellularly the signal is transmitted by Dsh and results in the inhibition of a cytoplasmic, Axin-nucleated complex containing casein kinase-1α, glycogen synthase kinase-3β, and the adenomatous polyposis coli protein, in addition to other proteins (7–9). In the quiescent situation, this complex serves to constitutively phosphorylate the soluble pool of β-catenin, resulting in its ubiquitination and subsequent rapid degradation by the 26 S proteasome (10). Upon Wnt stimulation, β-catenin thus becomes stabilized and acts in the nucleus as a transcriptional coactivator of target genes by interacting with high mobility group box transcription factors of the Lef1/Tcf subfamily (11, 12).

Embryos from Xenopus have been remarkably useful in elucidating several of the mechanistic intricacies at play during canonical Wnt signaling. Xenopus also became the first vertebrate organism in which a major role for canonical Wnt signaling was unveiled, i.e. the specification of the dorsal territory in the pregastrula embryo. Despite other, more recent insights into the functional significance of canonical Wnt signaling in a number of biological systems, its full spectrum of cellular and supracellular implications still holds many aspects to be uncovered. Traditional knock-out models in vertebrates can only partially succeed in building our understanding most notably because of the frequent occurrence of functional redundancy of pathway components. Morpholino-mediated gene knock-down strategies in Xenopus only permit the evaluation of phenotypes during the first days of development, and potential early developmental perturbations can obfuscate later phenotypes. Here we describe an alternative loss-of-function approach that makes use of transgenically modified Xenopus laevis embryos in which transcription of Lef1/Tcf-β-catenin target genes can be blocked in a temporally controlled way. Besides its clear potential for advancing our comprehension of canonical Wnt signals during embryogenesis, our transgenic system opens the opportunity to identify transcriptional targets within the primate end of the macromolecule. Developing organism.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Standard cloning techniques were used to generate the different constructs. Plasmid fragments were amplified by low cycle number PCR using Vent DNA polymerase (New England Biolabs, Beverly, MA). The DNA-binding domain of mouse Lef1 was recovered from LefΔN-βCTA described by Vleminckx et al. (13). Plasmids containing the hormone-binding domain of the human glucocorticoid receptor (GR) and the repression domain of Drosophila Engrailed

* This work was supported by grants from the Belgian Federation Against Cancer and the Interuniversitaire Attractiepols. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** The abbreviations used are: GR, hormone-binding domain of the glucocorticoid receptor; EnR, repression domain of Drosophila Engrailed; EF-1α, elongation factor-1α; EGFP, enhanced green fluorescent protein; DX, dexamethasone; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; RT, reverse transcription; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; UAS, upstream activating sequence.

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
(EnR) were generous gifts from Andreas Hecht and Daniel Kessler (14), respectively. A multiple element transgenesis vector was built onto the backbone of pBlueScriptISK (Stratagene). The X. laevis EF-1α promoter and the carp El1 basal promoter preceeded by a 14-mer of UAS Gal4-binding sites were isolated from the plasmids EF-GVP and UG described by Koster and Fraser (15) and kindly provided by these authors. In addition, EF-GVP was used for generating the Gal4-VP16-GR fusion. The basic human E-cadherin promoter described by Comijn et al. (16) was used to drive EGFP expression. The different coding constructs were all demarcated at their 5′ end by the SV40 late polyA site and were extensively verified by sequence analysis. More details regarding the cloning strategy can be obtained upon request.

TOP/FOPFLASH Reporter Assays—The capacity of the different constructs to inhibit canonical Wnt signaling was measured upon transfection of SW480 cells using the TOP/FOPFLASH reporter plasmids (17). An artificial Lef/Tcf-responsive promoter followed by the luciferase gene is present in the TOPFLASH reporter, while the FOPFLASH control plasmid contains a mutated, nonresponsive promoter. SW480 cells were grown at 37 °C in L15 medium supplemented with 10% fetal calf serum, 2 mM L-Gln, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were seeded in 24-well plates and transfected in triplicate using FuGENE 6 transfection reagent (Roche Applied Science). Transfection mixtures were mass-equalized using empty pCS2 and grown at 18 °C in the absence or presence of 10-5 M DX. Measures were carried out in a Topcount NXT luminometer (Packard Instrument Co.).

RT-PCR Analysis—RT-PCR analysis was done on individual embryos. Total RNA was extracted using RNAzol reagent (Tel-Test). First strand cDNA synthesis was done using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamer primers in a 20-μl reaction volume containing half of the RNA preparation. A cDNA aliquot of 1 μl of RNA was amplified using AmpliTaq Gold DNA polymerase (Applied Biosystems) in the presence of [α-32P]dCTP label. Sequences of forward and reverse primers were as follows (5′ to 3′ orientation): CAGATGTCGTGGATTGAGTCG and ACTGCTCTGAGAAGTGAGAGTCTCTAG for EF-1α, TGGTCTCTACCCGACTGTA and TCTCTGGTACGAGGCTACT for Siamois, GTGAGCTCAGCTTGGCAGT and ACAGA-GCCAACTCTTGATG for Xnr3, TTCTCAGGTCCGAGCTC and TTCTTGAACGTGGGCGG for En-2, GCCCACTCATCATTTTCAT and GGAATACGATGAAGCCTCA-CTTTGAAGTGGTCGCG for Engrailed, and the hormone-binding domain of the human glucocorticoid receptor. The high mobility group box present in Lef1/Tcf transcription factors consists of a highly conserved DNA-binding domain and mediates recognition of a conserved consensus sequence in the promoter region of target genes (25, 26). The incorporation of a steroid-binding domain aims at keeping the corresponding protein quiescent until an appropriate ligand liberates its functional activity (27). Most often, the transcription activator properties of the chimeric fusion proteins are analyzed by measuring the strength of the repression that was incited. In addition, varying conditions regarding the cloning strategy can be obtained upon request.

RESULTS

An Optimized Inducible Repressor of Target Genes of Lef1/Tcf-dependent β-Catenin Signaling—To block transcriptional activation of target genes of canonical Wnt signaling in a way that would permit temporal control we resorted to the use of a hormone-inducible fusion composed of the high mobility group box of mouse Lef1 (LefΔN), the repression domain of Drosophila Engrailed, and the hormone-binding domain of the human glucocorticoid receptor. The high mobility group box present in Lef1/Tcf transcription factors consists of a highly conserved DNA-binding domain and mediates recognition of a conserved consensus sequence in the promoter region of target genes (25, 26). The incorporation of a steroid-binding domain aims at keeping the corresponding protein quiescent until an appropriate ligand liberates its functional activity (27). Most often, the transcription activator properties of the chimeric fusion proteins are analyzed by measuring the strength of the repression that was incited. In addition, varying conditions regarding the cloning strategy can be obtained upon request.
degrees of background repression could be detected when DX was absent. Based on this comparative analysis, we chose to proceed with EnR-LefAN-GR since this variant displays a low level of background activity combined with a fairly strong functional activation. This favorable fusion was then further optimized by mutational inactivation of the TAF-2 motif in the GR domain (Glu to Ala substitution at position 755). This motif has the capacity to recruit transcriptional coactivators (28, 29), which may operate in quenching the activity of the EnR repression domain. The resulting mutant fusion, referred to as EnR-LefAN-GR<sup>755A</sup>, was found to exert superior repression coupled with a strong functional inducibility.

**EnR-LefAN-GR<sup>755A</sup> Strongly Inhibits Lef1/Tcf-β-Catenin Signaling in Xenopus Embryos**—We then examined whether our optimized fusion could also inhibit canonical Wnt signaling in Xenopus embryos. In blastula stage embryos, dorsal activation of the maternal Wnt pathway prevents the initiation of a ventral fate program. Following dorsal microinjection with EnR-LefAN-GR<sup>755A</sup>, embryos were kept in culture with or without DX. The powerful inhibitory effect of EnR-LefAN-GR<sup>755A</sup> on canonical Wnt signaling was evidenced by severe ventralization of the phenotype in the presence of DX (Fig. 1B). At the dose used, no signs of ventralization were observed when DX was omitted. DX-dependent inhibition of canonical Wnt signaling in injected embryos was further confirmed by demonstrating repression of the transcriptional targets Siamois and Xnr3 (Fig. 1C). These results also highlight the fact that although the fusion incorporates the DNA-binding domain of Lef1, its capacity to inhibit target genes is not subordinate to the particular type of endogenous Lef1/Tcf usage, as Siamois is controlled via Tcf3 (30).

**Design and Integration of a Transgene Vector Based on EnR-LefAN-GR<sup>755A</sup>**—Clearly, integration of EnR-LefAN-GR<sup>755A</sup> as a transgene strongly requires that the activity of the fusion can be blocked firmly until DX is added. We were uncertain whether this aspect was sufficiently bolstered to permit constitutive expression of EnR-LefAN-GR<sup>755A</sup> as a transgene. Despite the strong inducibility of EnR-LefAN-GR<sup>755A</sup>, a ~2-fold reduction of TOPFLASH reporter activity was observed upon transfection when DX was absent (Fig. 1A). This partial leakiness in DX dependence was also observed in *Xenopus* embryos upon microinjection with EnR-LefAN-GR<sup>755A</sup> RNA in excess of a certain threshold dose (not shown).

To warrant leak-proof control of the fusion, we extended the inducibility of EnR-LefAN-GR<sup>755A</sup> by DX to its transcriptional level by incorporating a Gal4-VP16/GR/UAS induction loop. We created a single, composite transgene vector featuring all the required sequences (Fig. 2). A fusion encoding a DX-inducible Gal4-VP16-GR transactivator was placed under transcriptional control of the ubiquitously active *Xenopus* EF-1α promoter. The minimal carp E1b promoter preceded by a multimer of 14 Gal4-binding UAS elements was used to drive expression of EnR-LefAN-GR<sup>755A</sup>. Because amphibian transgenesis gives rise to a mixed population of transgenic and non-transgenic post-transplantation embryos, an EGFP expression cassette containing a small size, basic derivative of the human E-cadherin promoter (16) was built into this vector as well, thus providing a screenable marker and allowing verification of proper non-mosaic integration. Transgenic embryos were usually characterized by a faint gloss of fluorescence. A clear advantage of the basic E-cadherin promoter is its early onset of activity in the superficial tissue layers, enabling identification of *bona fide* transgenic F<sub>0</sub> embryos already at midgastrulation. In early embryos, EGFP appears to be expressed in most tissues. Later, EGFP fluorescence acquires a more tissue-specific character, while its overall intensity seems to decline.

We then verified whether EnR-LefAN-GR<sup>755A</sup> was firmly quenched by this double mode of dependence on DX. A problem inherent to the nuclear transplantation technique in *Xenopus* transgenesis is the fairly high frequency of defects that arise during gastrulation of post-transplantation F<sub>0</sub> embryos. Although often resulting in early mortality, these gastrulation defects also yield a sundry collection of malformations at later embryonic stages, which can seriously obfuscate the transgene-elicited phenotype. The physical linkage of EnR-LefAN-GR<sup>755A</sup> with a screenable EGFP marker provides a useful aid to distinguish specific defects from spontaneous anomalies. To ascertain whether our inducible system was leak-proof, transgenic, healthy end-gastrula F<sub>0</sub> embryos were singled out at random and followed through development for the appearance of phenotypic defects. We focused particularly on embryos characterized by strong fluorescence, since these would be more susceptible to functional leakiness of the construct because of multiple transgene copies or because of integrations in transcriptionally hyperactive loci. However, no phenotypic abnormalities could be detected. In fact, transgenic embryos could be raised to well beyond the limb bud stage (see Supplemental Fig. S2). To the extent that these external inspections allow dependable inference, we conclude that transgenically integrated EnR-LefAN-GR<sup>755A</sup> does not perturb normal premetamorphic development as long as DX is excluded from the medium.
DX Induces Expression of EnR-LefΔN-GR755A in Transgenic Embryos—To test proper induction of EnR-LefΔN-GR755A expression upon treatment with DX, transgenic F₀ embryos were examined by Western blot analysis. As shown in Fig. 3, expression of EnR-LefΔN-GR755A was clearly induced upon culturing with DX. No clear protein band could be detected in the absence of DX. It is difficult to predict whether this low background expression makes additional, protein-intrinsic DX dependence of EnR-LefΔN-GR755A dispensable. It is important to keep in mind, however, that in nonestablished F₀ embryos carrying random transgene integrations, individual differences in background expression are likely to exist as was also demonstrated recently using a doxycycline-inducible system (31). In light of this, we judge that the post-translational inducibility of EnR-LefΔN-GR755A adds a secure fail-safe.

DX Treatment Causes Repression of Lef1/Tcf-β-Catenin Targets in Transgenic Embryos—To prove that Lef1/Tcf-β-catenin-mediated gene induction can be blocked in EnR-LefΔN-GR755A transgenic F₀ embryos, we sought to demonstrate altered expression of known downstream effector genes by RT-PCR analysis. Beyond the gastrula stage of Xenopus development, only the En-2 marker gene of the midbrain-hindbrain boundary has so far been identified as a direct transcriptional target (32). As shown in Fig. 4A, addition of DX at the end-gastrula stage nearly completely abolished En-2 expression. A second candidate we investigated was the tyrosine kinase receptor EPH-B3, which was identified as a downstream target of canonical Wnt signaling in the mammalian intestine and in ovarian tumors (33, 34). In addition, its embryonic expression pattern has been partially described in chicken (35). Down-regulation of EPH-B3 in the presence of DX was less prominent but nevertheless clearly appreciable when compared with a set of control genes (Fig. 4B). Together, these results validate the use of our inducible Wnt-inhibitory transgenic system.

Induction of EnR-LefΔN-GR755A in Transgenic Embryos Abrogates Embryonic Development—Functional exploration of canonical Wnt signaling during Xenopus development has been almost exclusively conducted by classical microinjection experiments at the early blastula stage, essentially constraining thus the time frame and target region of intervention. Our transgenic system now permits the assessment of the phenotypic repercussions stemming from inhibition of canonical Wnt signaling in variously aged transgenic embryos. For these phenotypic examinations, we selected F₀ embryos that appeared perfectly normal and were not younger than the end-gastrula stage to avoid interference from the spontaneous defects that may arise from the nuclear transplantation procedure. Starting from a specified embryonic stage, both kinds of post-transplantation F₀ embryos, i.e., EGFP⁺ and EGFP⁻, were grown in parallel in the continuous presence of DX and monitored further through development. On the rightful assumption that absence of fluorescence indeed attests to absence of the transgene, no effects were to be expected in the EGFP⁻ F₀ individuals, and these embryos thus served as a reference control.

Following a delay required for expression and functional impact of EnR-LefΔN-GR755A, we observed that exposure of transgenic embryos to DX caused a dramatic and general abrogation of normal embryogenesis at each of several stages examined and produced a lethal outcome (Fig. 5). Some variation in the severity of the phenotypic picture that came forward could be discerned, but this could well be correlated with the intensity of the fluorescence signal. A common characteristic of these embryos was the apparent arrest of developmental progression, manifested in stagnation of body size and failure to form newly acquired structural features stereotypical for normal embryonic growth. During early development, the embryo rapidly proceeds through a series of intense morphogenetic changes. Concomitantly, addition of DX during these early stages rapidly impelled visible defects shortly followed by dis-integration of the embryos. At later stages of DX treatment, we observed a growing delay in the emergence of gross abnormalities, concurrent with the equal deceleration of altering general morphology in normal embryos. Nonetheless, transgenic embryos of more advanced stages were also fated to become arrested and to die off as a result. Interestingly, some of the phenotypic repercussions, such as the inhibition of tail outgrowth or the absence of eyes, provide a direct link with earlier descriptions of Wnt involvement or seem to recapitulate phenomena observed by others (36, 37). In sum, we believe that the encountered broad scale defects bear compelling testimony to a widespread and incessant importance for canonical Wnt signaling during animal embryogenesis.

Induction of EnR-LefΔN-GR755A Reduces Cell Proliferation and Increases Apoptosis in Transgenic Embryos—Several sources of evidence indicate that canonical Wnt signals can stimulate the proliferative capacity of certain cells. It was therefore important to examine whether transgenic embryos expressing activated EnR-LefΔN-GR755A were affected at the level of cellular proliferation. Following incubation with or without DX, intestinal organ explants were dissected and exposed to BrdUrd incorporation. Sections of the gastrointestinal tract were largely deprived of S phase cells as a consequence of DX treatment (Fig. 6, A and B). This effect was apparently not
observed throughout the embryo as other organs, such as the liver, were not affected at the stage examined (Fig. 6B). It must be noted that these assays cannot distinguish between cell cycle arrest or elimination of actively dividing cells because of programmed cell death. Others have demonstrated that deregulated Wnt signaling can indeed affect cellular survival (38–41). In support of this, whole-mount TUNEL assays revealed a similar general impairment of morphogenesis was observed upon treatment of late neurula embryos (stage 23), which did not develop discernable eyes. A and B, at these early stages of DX treatment, death by disintegration was rapid. C, transgenic tailbud embryos (stage 31) were characterized by the inhibition of tail outgrowth, the subsistence of an undifferentiated gut system, and large edematous dilatations. D, similar manifestations were observed when young tadpoles (stage 39) were treated.Embryos were able to survive increasingly longer during the DX treatment. It was noted that this lag had been shown at stages of development. A–D, DX-treated, control EGFP- post-transplantation embryos develop normally at least for the first 5–6 days. Later, DX effects do become apparent, but these symptoms were mild and not related to the transgene and were equally observed in naturally fertilized wild type embryos (not shown). st., stage.

**DISCUSSION**

Loss-of-function analysis in various model organisms has often yielded key insights into fundamental aspects of cellular dynamics, tissue patterning, and embryonic morphogenesis. In this work, we established a transgenic system in *X. laevis* aimed at the conditional blocking of canonical Wnt signaling at its nuclear end point, i.e. the transcriptional activation of target genes by Lef1/Tcf-β-catenin complex formation. For this purpose, we transgenically integrated a DX-controllable fusion designed to act as a transcriptional repressor. Examination by RT-PCR analysis demonstrated that treatment of transgenic embryos with DX led to transcriptional repression of known target genes. Thus, endogenous signaling via the canonical Wnt pathway can be blocked conditionally in these transgenic embryos at any time point of premetamorphic development. Wnt/β-catenin signaling has been shown to play essential roles at many different times and locations within the developing embryo. Our results further add compelling support to the crucial and widespread importance of this pathway during embryogenesis. At each of several stages examined, transgenic embryos became severely affected on a gross scale when cultured in the presence of DX. External examination revealed that these embryos largely failed to continue with phenotypic differentiation and became severely impeded in their body outgrowth. Thus, chronic inhibition of endogenous canonical Wnt signaling throughout the embryo abrogates morphogenesis in general. Recent and intriguing evolution in our perception of canonical Wnt signaling has been its implication as a potent growth stimulus for the clonal expansion of stem cells and...
certain types of progenitor cells (42–45). This notion could possibly provide important clues to explain some of the aspects underlying the phenotypic outcome observed in this study. Obviously, progenitor cells of varying multipotency must reside in many, if not all, embryonic tissues to maintain a status of active growth and to initiate pedigrees of new lineages of differentiation. Paninhibition of canonical Wnt signaling could conceivably interfere with the capacity of a broad range of these founder cells to undergo self-renewal, by which they would become rapidly depleted. One can easily imagine that a condition of this kind would drastically undermine the elaboration of a developmental program, accompanied by an increase in apoptosis. Yet, a general inverse relationship between canonical Wnt signals and programmed cell death has many other interaction partners, including transcription. This avoids the risk of interfering with other signaling in relation to apoptosis is more ambiguous. In supposal, an incom- 

ment status of canonical Wnt signaling requires a compatible activation of the pathway can result in elevated apoptosis as well (47, 48). One could postulate that a reversal in the activation status of canonical Wnt signaling requires a compatible cellular condition, dictated by the presence or absence of other factors, be it intrinsic or extrinsic. In this supposition, an incompatible switch in canonical Wnt signaling, due to experimental deregulation, could potentially trigger an apoptotic program. Such a model could possibly reconcile both classes of data. A clear advantage of our transgenic system over existing approaches is its direct impact at the level of target gene transcription. This avoids the risk of interfering with other signaling events that branch out from upstream components of the canonical Wnt pathway. For example, it has been shown that β-catenin, apart from its well documented role in cell adhesion, has many other interaction partners, including transcription factors such as Pitx2 or certain Sox members (49, 50). Targeted ablation of β-catenin could thus well affect a broader scale of phenomena rather than just inhibition of Lef1/Tcf-responsive genes. Another strategy to directly block transcriptional activation by β-catenin was reported by Tepera et al. (41) and involves the transgenic expression of a chimera that couples the Lef1/Tcf interaction domain of β-catenin to EnR, but this fusion has so far not been used in an inducible setting. It will be of interest to direct expression of EnR-LefαN-GR(25A) in specific tissues by substituting the ubiquitously active EP-1a promoter. Furthermore, recent work using doxycycline as a small molecule inducer in transgenic Xenopus has demonstrated the feasibility of reversible interference during a defined developmental episode, simply by washing out of the ligand (31). These additional possibilities hold out the prospect to exert full spatial-temporal control over this important pathway. In addition, we believe that these transgenic embryos constitute a powerful source for analysis of differential gene expression to track down transcriptional targets in a variety of tissues and organs.

Acknowledgments—We are grateful to Andreas Hecht, Daniel Kessler, Reinhard Köster, and Geert Berx for sharing expression plasmids. The TOP/FOPFLASH reporter plasmids were kindly provided by Hans Clevers and Marc van de Wetering. We thank A. Bredan for editorial assistance.

REFERENCES
1. Cadigan, K. M., and Nusse, R. (1997) Genes Dev. 11, 3286–3305
2. Polakis, P. (2000) Genes Dev. 14, 1837–1851