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# Anterior axis duplication in *Xenopus* induced by the over-expression of the cadherin-binding protein plakoglobin

**(desmosomal cadherins/mesoderm induction/nuclear localization/*Xenopus laevis*)**

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**ABSTRACT** **Plakoglobin interacts with both classical and desmosomal cadherins. It is closely related to *Drosophila* armadillo (*arm*) gene product; *arm* acts in the wingless (*wg*)-signaling pathway to establish segment polarity. In *Xenopus*, homologs of *wg*—i.e., *wnts*, can produce anterior axis duplications by inducing dorsal mesoderm. Studies in *Drosophila* suggest that *wnt* acts by increasing the level of cytoplasmic armadillo protein (arm). To test whether simply increasing the level of plakoglobin mimics the effects of exogenous *wnts* in *Xenopus*, we injected fertilized eggs with RNA encoding an epitope-tagged form of plakoglobin; this induced both early radial gastrulation and anterior axis duplication. Exogenous plakoglobin accumulates in the nuclei of embryonic cells. Plakoglobin binds to the tail domain of the desmosomal cadherin desmoglein 1. When RNA encoding the tail domain of desmoglein was coinjected with plakoglobin RNA, both the dorsalizing effect and nuclear accumulation of plakoglobin were suppressed. Mutational analysis indicates that the central arm repeat region of plakoglobin is sufficient to induce axis duplication and that this polypeptide accumulates in the nuclei of embryonic cells. These data show that increased plakoglobin levels can, by themselves, generate the intracellular signals involved in the specification of dorsal mesoderm.**

Wnts are a family of secreted proteins involved in intercellular signaling (1, 2). In *Drosophila*, the wingless (*wg*) protein, a defining member of the wnt protein family, is involved in the determination of embryonic segment polarity (see ref. 3). In vertebrates *wnts* play a critical role in the development of the central nervous system (4). The role of *wnts* in early vertebrate development has been most intensively studied in the clawed frog *Xenopus* (see refs. 5 and 6). Of the *Xenopus wnts* (*Xwnts*) identified *Xwnt-5A, -8,* and *-11* are present in the blastula-stage embryo. Starting with the initial observation of McMahon and Moon (7), it is clear that ectopic expression of the mammalian protooncogene *int-1* (the other founder of the *wnt* family), *Xwnt-1, -8,* or *-11* (8), and the *Drosophila wg* protein (9) can induce the formation of ectopic dorsal mesoderm. Through its ability to act as an inducer, this ectopic dorsal mesoderm produces duplications in the anterior region of the later-stage embryo.

Genetic analysis of the *wg* signal-transduction pathway has identified a number of the gene products involved; *arm* is the terminal member of this pathway and acts to stabilize the expression of engrailed (*en*) (see refs. 3 and 10–12). Armadillo protein (arm) is closely related to the vertebrate cadherin-binding proteins β-catenin and plakoglobin (γ-catenin) (13, 14). In vertebrate cells the "classical" cadherins, found at adherens junctions, interact through plakoglobin or β-catenin, and additional accessory proteins, with the cell's microfilament system (15). The desmosomal cadherins interact through plakoglobin and a different set of accessory proteins, with intermediate filaments (16). Plakoglobin is unique in that it interacts with both classical and desmosomal cadherins (see refs. 14 and 17) and is present at both types of cell–cell adherence junctions (18).

There are two models for the role of arm-like proteins in the *wnt* signaling pathway: either they act as part of the cellular adhesion junctions or they act as "free" components. Evidence in support of the later model comes from studies by Peifer *et al.* (12, 19) that show that expression of *wg* leads to an increase in cytoplasmic arm. In cultured vertebrate cells, exposure to wnt is reported to increase the levels of plakoglobin and β-catenin (20, 21). Given that the ectopic expression of *wnts* (see above) and the injection of anti-β-catenin antibody (22) both induce axis duplication in *Xenopus* embryos, it was a natural question whether simply increasing the level of plakoglobin could induce axis duplication. To answer this question, we injected plakoglobin RNA into fertilized eggs and found that it induces the formation of secondary body axes. To examine the mechanism of this effect, we coexpressed the cytoplasmic tail domain of the desmosomal cadherin desmoglein, which is known to bind to plakoglobin (23–25). The results of these experiments show that the simple overexpression of plakoglobin induces axis duplication in *Xenopus*.

## METHODS

**Construction of Plakoglobin and Desmoglein Tail Plasmids.** PCR was used to isolate the full coding region or the central repeat domain (amino acids 118–680) of a *Xenopus* plakoglobin cDNA (26) and the cytoplasmic domain from a human desmoglein-1 cDNA (27) (Fig. 1). Amplified DNAs were subcloned into the pT7*mycVimTail plasmid, which adds the myc-derived sequence MEQKLISEEDLN to the N terminus of the final polypeptide. pT7* is derived from the pSP64T plasmid by replacing the SP6 RNA polymerase promoter with the T7 promoter sequence (see ref. 28). The final constructs were analyzed by restriction digest and through the use of a coupled transcription/translation system (Promega). The myc-plakoglobin plasmid generated a polypeptide of apparent $M_r$ 85,000, close to its calculated $M_r$ of 83,044. The apparent $M_r$ of the mycDsgTail polypeptide was ≈70,000, somewhat larger than its calculated $M_r$ of 51,673.

**RNA Synthesis and Microinjections.** Capped RNAs were synthesized from linearized plasmids using the Ambion mMessage mMachine *in vitro* transcription kit following the manufacturer's instructions. Capped RNA (20 nl at 250 pg/nl and lower) was injected into fertilized eggs, as described (29). In coinjection experiments, RNAs were mixed so that each was present at the same final concentration.

**Analysis of Injected Embryos.** At stage 13, groups of embryos were homogenized in SDS sample buffer, and an

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Abbreviations: arm, armadillo protein; *Xwnt*, Xenopus *wnt*; CRR, central *arm* repeat region.

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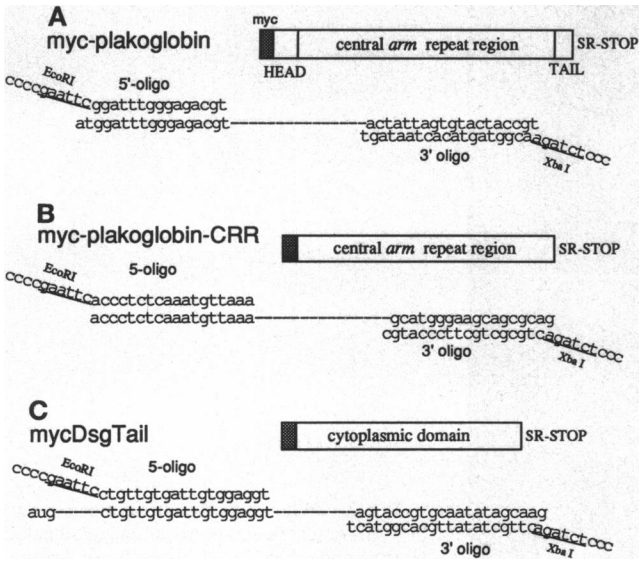


FIG. 1. A schematic of the constructs used. PCR was used to amplify the full coding region of *Xenopus* plakoglobin (*A*), the central arm repeat region of plakoglobin (amino acids 118–680) (*B*), and the cytoplasmic domain of human desmoglein 1 (beginning at amino acid 577) (*C*). The 5′ primers include sites used for cloning into the pT7*myc plasmid (28). Five to 10 cycles of PCR were used to amplify the DNA region of interest; each plasmid was tested with a coupled transcription/translation system to determine that a polypeptide of the appropriate molecular weight was synthesized (data not shown).

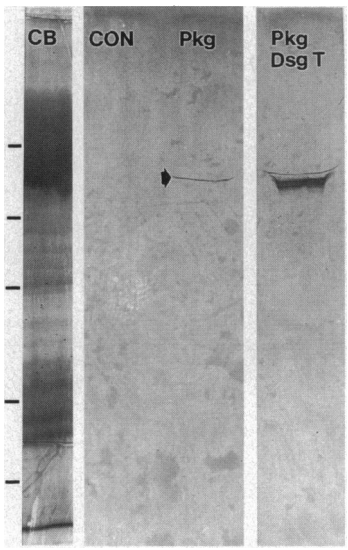


FIG. 2. Immunoblot analysis of embryos injected with myc-plakoglobin RNA. The equivalent of a single embryo stage-13 embryo (stages defined by ref. 32) was analyzed by SDS/PAGE–immunoblot. Lane marked CB displays the proteins loaded, as visualized by Coomassie brilliant blue staining. In uninjected embryos (CON) no anti-myc immunoreactive polypeptides are visible. In embryos injected with myc-plakoglobin RNA (Pkg), 9E10 antibody stained a single polypeptide (arrow), which runs at the leading edge of the large yolk band. Coexpression of mycDsgTail and myc-plakoglobin (Pkg Dsg T) had no apparent effect on the accumulation of myc-plakoglobin. The positions of molecular mass markers (83 kDa, 62 kDa, 47.5 kDa, 32.5 kDa, and 25 kDa) are indicated at left.

aliquot, equivalent to a single embryo, was analyzed by SDS/PAGE–immunoblot using the anti-myc antibody 9E10 (30) and peroxidase-conjugated secondary antibodies; bound antibody was visualized by using either the diaminobenzidine reaction or enhanced chemiluminescence (Amersham). For whole-mount confocal microscopy, stage-13 embryos were prepared as described by Cary and Klymkowsky (31) using the anti-myc antibody 9E10 and fluorescein-conjugated secondary antibody. Video images of various stages of embryonic development were captured on an Apple Macintosh Ci computer using a Panasonic charge-coupled device camera and a "Digivideo" video digitizer board, as described (29). Images were manipulated using the National Institutes of Health IMAGE software package.

## RESULTS

**Injection of Plakoglobin RNA Causes Axis Duplication.** Capped RNA encoding myc-plakoglobin was injected into the animal hemisphere of fertilized eggs. Immunoblot (Fig. 2) and whole-mount immunocytochemical analyses (see below) indicate that the polypeptide accumulates in the embryo and could be detected past the end of gastrulation. Myc-plakoglobin RNA injection had no obvious effects on cellular morphology or adhesion through the early blastula stage of embryonic development (data not shown). As gastrulation begins, however, a distinctive phenotype becomes apparent—namely, an acceleration in the formation of the blastopore in myc-plakoglobin RNA-injected embryos, i.e., the blastopore appeared early and was more radially complete, compared with control-injected embryos (Fig. 3 *A* and *B*). This result is quite different from the normal slight (≈0.5 stage) delay often seen in antibody or RNA-injected embryos (29).

Embryos injected with >5 ng of myc-plakoglobin RNA often failed to gastrulate; the blastopore, which formed apparently normally, stopped closing at ≈stage 11, and the yolk plug was extruded (data not shown). Embryos injected with 5 ng (or less) of myc-plakoglobin RNA passed through gastrulation successfully and displayed axial duplication at high frequency (Table 1). Duplications ranged from the formation of two distinct heads to the duplication of the cement gland only (Fig. 3). Early symmetrical gastrulation can be taken as a sign of embryonic dorsalization (33). The appearance of anterior duplications in myc-plakoglobin RNA-injected embryos supports this conclusion.

**Coexpression of the DsgTail Polypeptide Suppresses Plakoglobin-Induced Axis Duplication.** Plakoglobin binds to the cytoplasmic domain of the desmosomal cadherin desmoglein 1 (23–26). To determine whether the dorsalization/axis duplication effects of plakoglobin occur in the presence of excess desmoglein tail domain, we coinjected myc-plakoglobin RNA together with RNA encoding a myc-tagged version of the human desmoglein-1 cytoplasmic tail domain (mycDsgTail) (Fig. 1*C*). Even though the amounts of RNA injected were similar and both RNAs have identical 5′ and 3′ untranslated regions and are identical in the region of translation initiation, the mycDsgTail polypeptide consistently accumulates to higher levels than does myc-plakoglobin, as determined by immunoblot (Fig. 2). In contrast to studies that indicate that the interaction between plakoglobin and desmoglein tail stabilizes plakoglobin in cultured cells (34), we found no apparent effect of the presence of mycDsgTail on the stability of exogenous plakoglobin, as measured on a whole-embryo basis (Fig. 2). The co-injection of myc-plakoglobin and mycDsgTail RNAs suppressed dorsalized gastrulation (data not shown) and the anterior duplication phenotypes of exogenous plakoglobin (Table 1). Essentially identical results have been seen in four independent experiments.

**Subcellular Localization of Exogenous Plakoglobin.** To determine the subcellular localization of exogenous myc-plakoglobin polypeptide, RNA-injected embryos were stained in whole-mount and examined by confocal microscopy at stage 13. In the cytoplasm, the myc-plakoglobin polypeptide associated with the plasma membrane (Fig. 4*A*). In regions of high concentration, membrane-associated aggregates of myc-pla-

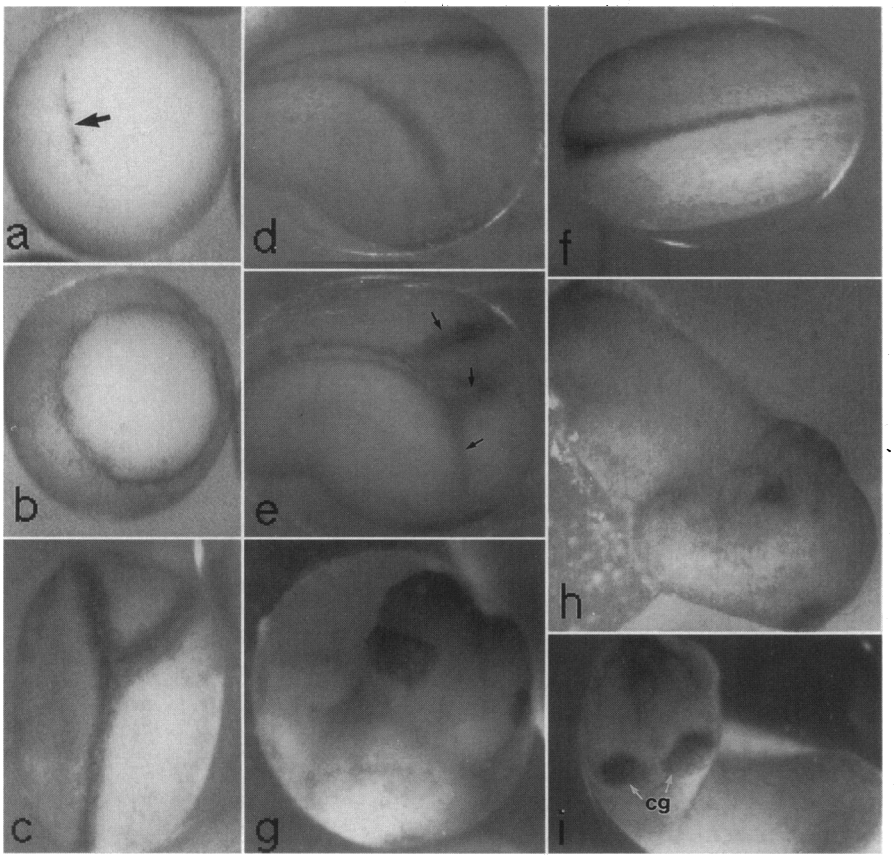


FIG. 3. When most control-injected embryos have just begun gastrulation, as signaled by the appearance of the dorsal lip (*a*, arrow), most myc-plakoglobin RNA-injected embryos have a nearly complete blastopore (*b*). Many myc-plakoglobin RNA-injected neural-stage embryos display partial (*c*) to complete (*d*) duplications of the anterior neural tube region. In rare cases, three neural axes are present (*e*, arrows). In control embryos, a single neural axis is present (*f*). By stage 25–28, duplications in the neural fold produce duplications of head structures (*g* and *h*). In the most subtle cases, only the cement gland (cg) appears to be duplicated (*i*).

koglobin were found (Fig. 4*A*). Surprisingly, given the published distribution of plakoglobin and arm within cells (12, 18, 19, 21), myc-plakoglobin staining was observed within the nuclei of embryonic cells in the form of punctate aggregates (Fig. 4 *A* and *B*).

When both myc-plakoglobin and mycDsgTail RNA were coinjected, the nuclear accumulation of labeled proteins was absent (Fig. 4 *D–F*); rather, staining was associated largely with the plasma membrane and the cytoplasmic surface of the nucleus (Fig. 4*F*). The membrane-associated staining ranged from rather uniform in appearance (Fig. 4 *E* and *F*) to irregular aggregates in regions of the embryo where staining was particularly intense (Fig. 4*D*). The presence of aggregates appears to reflect the interactions between myc-plakoglobin and the mycDsgTail because such aggregates were not seen in embryos injected with RNA encoding mycDsgTail alone. On its own, mycDsgTail was found primarily in the cytoplasm and associated with the cytoplasmic surface of the nuclear envelope and plasma membrane (data not shown).

Both β-catenin and plakoglobin have similar central arm repeat regions. In the case of β-catenin, this region is both necessary and sufficient to induce axis duplication (35). We therefore constructed a myc-tagged version of plakoglobin's central *arm* repeat region (CRR) (Fig. 1*B*). Coupled *in vitro* transcription/translation revealed the plasmid encoded a polypeptide of the expected molecular weight (data not shown). When RNA encoding the myc-plakoglobin-CRR polypeptide was injected into the animal hemisphere of fertilized eggs, it induced an axis-duplication phenotype, although the effect was not as pronounced as seen in plakoglobin (Table 1). Whole-mount immunocytochemistry of plakoglobin-CRR-injected embryos indicates that this polypeptide also accumulates in the nuclei of embryonic cells (Fig. 4*G*) but does so at lower levels than full-length plakoglobin.

Table 1. Axis duplication effects of plakoglobin

| | Single axis | Double axis | Not scoreable* |
|---|---|---|---|
| Water injected | 74 | 0 | 1 |
| Plakoglobin | 99 | 44 | 6 |
| Desmoglein tail | 31 | 0 | 16 |
| Desmoglein tail + plakoglobin | 33 | 4 | 18 |
| Plakoglobin-CRR | 165 | 18 | 16 |

*Embryos marked not scoreable are those in which the defect in gastrulation produced by myc-plakoglobin and/or mycDsgTail RNAs was sufficiently severe as to obscure subsequent developmental processes.

## DISCUSSION

Plakoglobin is a structural component of both desmosomes and adherens junctions (17, 18). It binds to the cytoplasmic tail domain of both desmosomal and classical cadherins and, through interactions with other proteins, mediates the anchorage of either microfilaments (at adherens junctions) or intermediate filaments (at desmosomes). In *Xenopus*, plakoglobin is present in the early embryo (26, 36). Given the role of the arm in the *wg*-signaling pathway of *Drosophila* and the effects of *wnts* and anti-β-catenin antibodies on mesoderm induction and axis formation in *Xenopus* (see above), it seemed likely that plakoglobin might also play a role in mesoderm induction. To test this hypothesis we injected plakoglobin RNA into fertilized eggs. The results are unambiguous: expression of exogenous *Xenopus* plakoglobin leads to both dorsalized gastrulation and anterior axis duplications (Fig. 3, Table 1).

To approach the mechanism by which plakoglobin acts, we examined the effects of coexpressing the tail domain of the desmosomal cadherin desmoglein 1. Desmoglein's tail binds to plakoglobin (23–26, 34), and a chimeric protein containing desmoglein's tail acts as a dominant mutation, disrupting desmosomes in cultured cells (37). In *Xenopus*, the expression of desmoglein tail suppressed the axis-duplication effects of

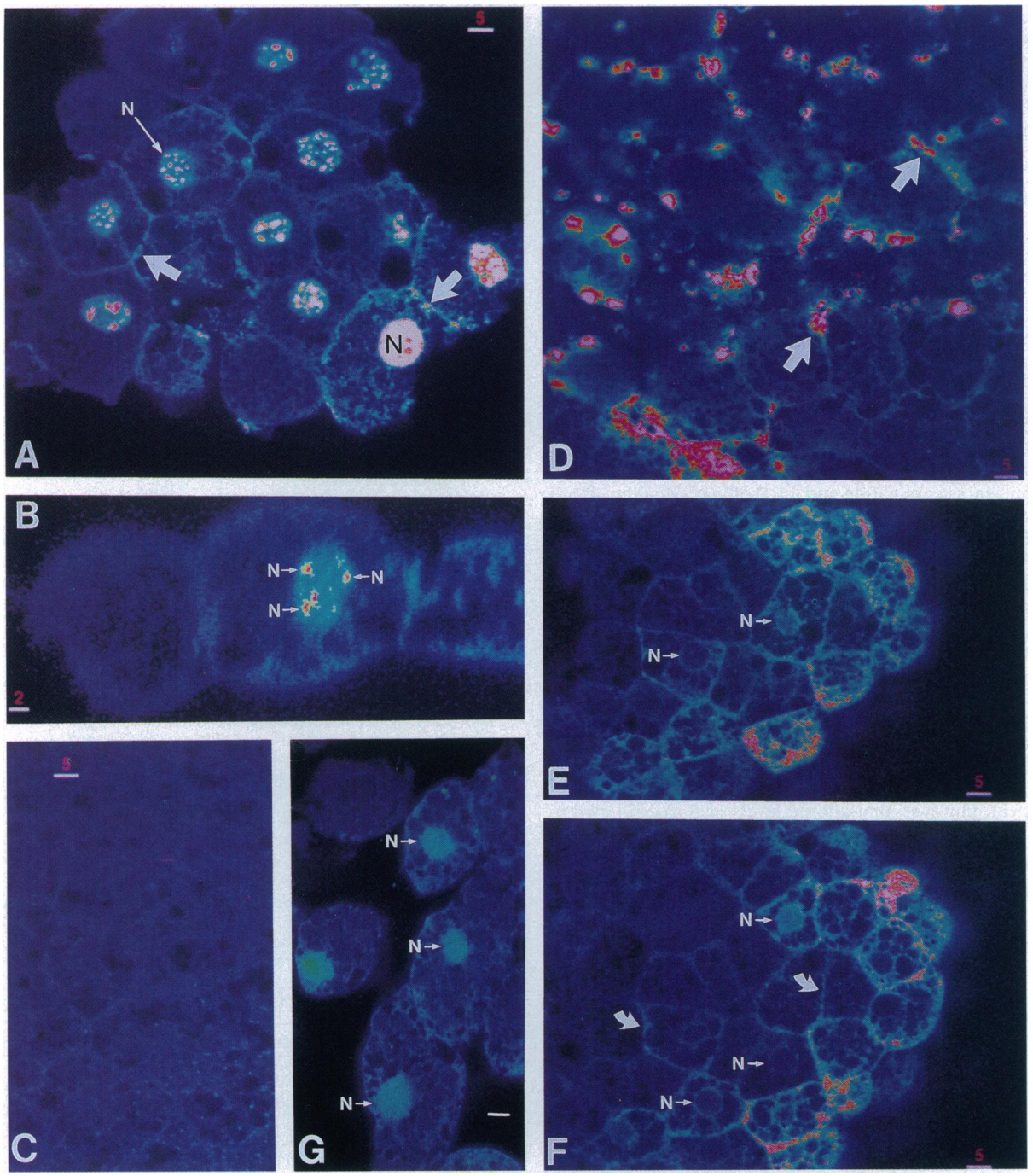


FIG. 4. Confocal microscopy revealed that exogenous plakoglobin was located both in the cytoplasm and within the nucleus of stage-13 embryonic cells (N) (*A* and *B*). The cytoplasmic staining was concentrated in the region of the plasma membrane (arrow). In the nucleus, myc-plakoglobin appears in punctate aggregates (N→). In control embryos, there was no significant 9E10 antibody staining of either the cytoplasm or the nucleus (*C*). In embryos injected with both myc-plakoglobin and mycDsgTail RNA (*D–F*), large 9E10 antibody-reactive structures were often seen; these aggregates appear to be associated with the plasma membrane (arrows). At lower levels, staining often appeared more uniform along the plasma membrane (arrows in *F*). Nuclear staining is either completely absent or very much reduced (nuclei are marked with N→). When RNA encoding the myc-plakoglobin-CRR polypeptide (*G*) was injected, staining with the 9E10 antibody was also found within the nuclei of embryonic cells. (Bars: *A*, 5 μm; *B*, 2 μm; *C–G*, 5 μm.)

exogenous plakoglobin (Table 1). We interpret this to mean that either plakoglobin must be a part of a functional cadherin complex, which the desmoglein tail/plakoglobin complex cannot form, to generate a cellular response, or that it is the free, non-cadherin-associated form of plakoglobin that acts as a signaling factor.

To examine whether exogenous plakoglobin was associated with the plasma membrane, we used whole-mount immunocytochemistry. In addition to the expected association with the cytoplasmic surface of the plasma membrane, we found that the exogenous plakoglobin also accumulated within the nuclei of embryonic cells. Its nuclear localization is unlikely to be due to the presence of the myc-epitope because (*i*) the myc-epitope does not itself contain a recognizable nuclear localization sequence (see ref. 38); (*ii*) other polypeptides containing this sequence do not enter the nucleus (31); and (*iii*) β-catenin, tagged with a hemagglutinin-derived epitope (DVPDYAS) also localizes to the nucleus (35). Moreover, in recent studies,

we have found that the localization of exogenous plakoglobin changes during development; it is cytoplasmic at stage 6 and begins to be found in nuclei starting at stage 7–8 (data not shown). Given the size of plakoglobin (≈84 kDa) and the fact that it is normally localized to adhesion junctions (18) or as a free form in the cytoplasm (12, 39), it seems likely that its nuclear localization is mediated by interactions with a "nuclear carrier" protein. The nature of this hypothetical transport factor is unknown.

The region involved in the binding of plakoglobin to desmoglein's tail domain has not been defined. The structurally similar β-catenin, however, interacts through its central *arm* repeat region with the cytoplasmic tail domain of classical cadherins (35, 40). Funayama *et al.* (35) have shown that this region is both necessary and sufficient to induce axis duplication in *Xenopus*. Not surprisingly, we find that the central *arm* repeat region of plakoglobin is also sufficient to induce axis duplication (Table 1), although it is less effective than is the entire plakoglobin polypeptide. Both the central *arm* repeat region of β-catenin (35) and plakoglobin (this work) accumulate in the nuclei of embryonic cells.

The nuclear accumulation and intracellular signaling effects of plakoglobin and β-catenin are reminiscent of the behavior of the cytoplasmic domain of the Notch protein. The Notch cytoplasmic domain binds to and represses the activity of basic helix–loop–helix proteins such as MyoD (41); moreover, when expressed as an isolated domain, the Notch cytoplasmic domain accumulates in the nucleus (41–44). As in the case of Notch protein (43), there is as yet no evidence that endogenous plakoglobin, β-catenin, or fragments derived from them localize to the nucleus. It is therefore unclear whether the nuclear localization of exogenous plakoglobin/β-catenin is functionally significant or whether it simply reflects the ability of these proteins to interact with factors that can be actively transported to the nucleus.

It is clear, however, that altered levels of β-catenin, plakoglobin, and classical cadherins influence dorsal mesoderm induction (23, 35, 45). Plakoglobin's "inductive" ability and the fact that it, like β-catenin, interacts with "classical" cadherins argues that the control of mesodermal differentiation and perhaps the differentiation of other tissues involve an interplay between these molecules.

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