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Technical Article

A hormone-dependent post-translationally regulated mutant for investigating type I cadherin function

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ABSTRACT Type I cadherins are Ca^{2+}-dependent cell adhesion molecules. Their function in early *Xenopus laevis* development has been extensively studied in recent years, by injecting synthetic mRNAs encoding dominant negative mutants with deletions of the extracellular domain into embryos. However, studies at post-gastrula stages have been hampered by the inability to progress through post-gastrula development in embryos expressing these mutant proteins. This problem has been partly overcome by injecting into a few targeted blastomeres in stage 6 N.F. embryos, but only restricted studies are possible with this technique. Several studies have made use of the hormone-binding domain (HBD), which is activated by hormones. In this study, we used this method to analyze the activity of dominant negative cadherins. We generated a mutant E-cadherin (ΔE-Cad, consisting of the cytoplasmic domain and transmembrane domain) fused to the hormone-binding domain of estradiol receptor (HBDER) and we validated this technique with functional analyses. The function of the mutant ΔE-HBDER was strictly dependent on hormone induction. This conditional mutant had the same effects and exerted the same dominant negative function as the corresponding constitutive mutant.

KEY WORDS: *type I cadherins, hormone-binding domain, Xenopus laevis*

Introduction

Type I cadherins are Ca^{2+}-dependent cell adhesion molecules widely expressed in *Xenopus laevis* from the early stages of development. EP/C-cadherin is the product of a maternal gene and is expressed throughout development (Choi *et al.*, 1990), E-cadherin is expressed from gastrulation onwards in the ectoderm (Levi *et al.*, 1991) and N-cadherin is expressed from neurulation onwards in restricted areas of ectoderm and mesoderm (Detrick *et al.*, 1990). Type I cadherins have a large extracellular domain, responsible for homophilic recognition, a transmembrane domain and a cytoplasmic tail, which is necessary for functional cell-cell adhesion by binding to β-catenin. If the extracellular domain is deleted, the resulting protein acts as a dominant negative mutant for all type I cadherins by recruiting the cytoplasmic pool of β-catenin and inhibiting its binding to endogenous type I cadherins (Kintner, 1992).

Cadherin function in *Xenopus laevis* development may be investigated *in vivo* by injecting synthetic mRNA encoding such mutant proteins at an early stage of development. This invariably has a dramatic effect due to inhibition of cell adhesion and consequent perturbation at mid-gastrulation (Broders and Thiery, 1995). This technique is therefore unsuitable for following the function of cadherins in post-gastrulation stages and in specific tissues. This problem can be partly overcome by injecting the mRNA into only a few blastomeres of stage 6 N.F. embryos, but this often results in the targeting of several tissues (Broders and Thiery, 1995). These drawbacks could be circumvented by time-specific expression. A successful approach has been reported for transcriptional factors, involving the post-translational regulation of inducible hybrids consisting of the protein of interest fused to a hormone-binding domain (HBD) (Picard *et al.*, 1988; Picard, 1993, 2000). The proteins are silent in the absence of the hormone and active in its presence. It has been suggested that the inactive state of the hybrid protein results from a steric hindrance mechanism involving the HSP90 protein. Binding of the hormone to its receptor leads to HSP90 release, thereby liberating the active site of the protein. This hypothesis is consistent with the observation that the

Abbreviations used in this paper: CMFM, calcium- and magnesium-free medium; ΔE-Cad, cytoplasmic dominant negative mutant of E-cadherin; HBD, hormone-binding domain; HBDER, hormone-binding domain of the estradiol receptor; HSP90, heat shock protein 90; NAM, normal amphibian medium; PBS, phosphate-buffered saline.

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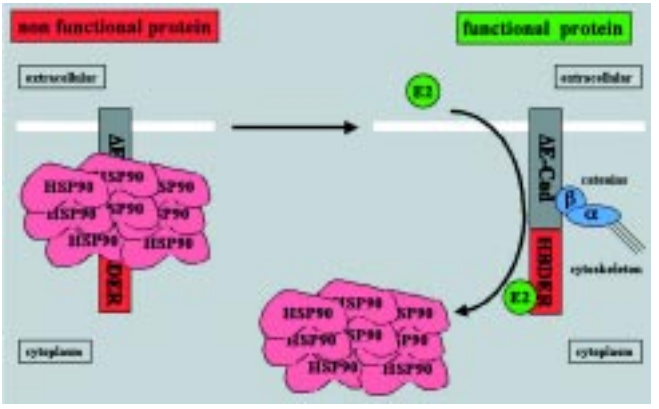

Fig. 1. Hypothetical model of ΔE-HBDER hybrid protein regulation. *In the absence of hormone induction, the binding site for β-catenin is concealed by HSP90, which forms a complex with HBDER. If β-estradiol (E2) is added, it binds to HBDER and displaces HSP90, freeing the β-catenin binding site, enabling the hybrid protein to exert its dominant negative effect.*

efficiency of repression depends on the intramolecular distance between the active site of the protein and the hormone receptor (Picard *et al.*, 1988). In recent years, HBD fusion proteins have been used extensively *in vitro* in cultured cells (Picard *et al.*, 1988; Hollenberg *et al.*, 1993; Picard, 1993) and *in vivo,* by the injection of synthetic mRNA in *X. laevis* (Kolm and Sive, 1995). Nevertheless, current studies remain limited to transcription factors and a few other intracellular molecules.

In this work, we investigated whether this system could be adapted to a mutant form of E-cadherin, deprived of the extracellular domain (ΔE-Cad), to achieve the required time specificity of the dominant negative function. This E-cadherin mutant is an intracellular protein and the β-catenin binding site is proximal to the C-terminal end of the cytoplasmic tail of E-cadherin (Ozawa *et al.*, 1990): these properties may fulfill the criteria for exploitation of the hormone induction system (the proposed working model is represented in Fig. 1).

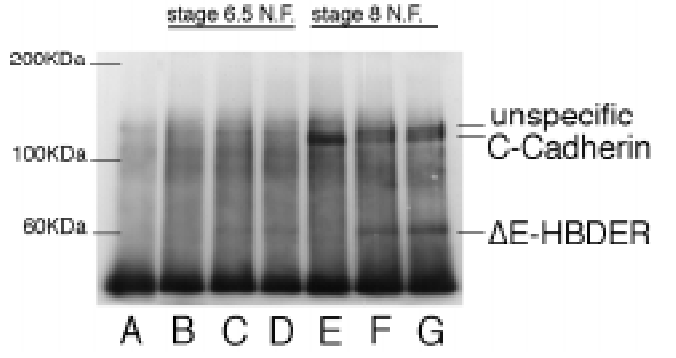

Fig. 2. The inducible construct is expressed from very early stages onwards. *Both endogenous C-cadherin and the ΔE-HBDER dominant negative mutant protein were detected by immunoprecipitation with a pan-cadherin antibody. Two bands were detected, of approximately 120 and 60 kDa in size, corresponding to C-cadherin and ΔE-HBDER, respectively. Lane* **(A)** *uninjected embryos incubated only with protein A-Sepharose, the band around 130 kDa results from non specific binding. Lanes* (B-D) *and* (E-G) *correspond to embryos collected at stage 6.5 N.F. (four hours post-fecundation) and at stage 8 N.F. respectively.* **(B,E)** *uninjected.* **(C,F)** *injected uninduced.* **(D,G)** *injected and induced.*

To test this system, we created a construct encoding a protein consisting of ΔE-cadherin fused to the ligand-binding domain of the estradiol receptor (ΔE-HBDER). We produced a synthetic mRNA from this construct, injected it into embryos and tested the expression and then the functioning of the mutant after hormone induction. We checked that β-estradiol had no collateral effects and that the uninduced hybrid protein had no activity. Functional analysis showed that the inducible system was effective after induction, that its function was similar to that of the constitutive dominant negative E-cadherin and that the dominant negative effect was due to recruitment of the cytoplasmic β-catenin.

Our *in vivo* analyses demonstrated that the inducible system could be applied to the dominant negative cadherin. Combined with targeted injections into stage 6 N.F. *Xenopus laevis* embryos, this system provides a powerful tool for studying cadherin function during numerous developmental processes.

Experimental procedures

Constructs and working model

The ΔE-Cad mutant was obtained by PCR amplification of the fragment containing the sequence encoding the transmembrane and cytoplasmic domain of E-cadherin (Broders and Thiery, 1995). This fragment was inserted into the PSP35N-Tag vector for mRNA synthesis *in vitro* (Dufour *et al.*, 1994). The inducible construct was obtained by the in-frame insertion, at the 3' end of ΔE-Cad, of the sequence encoding the ligand-binding domain of the estradiol receptor. Capped mRNA was produced with the Ambion kit (Ambion Inc., Austin, TX) and resuspended in water.

β-galactosidase mRNA was coinjected as a cell tracer: the coding sequence was inserted into the pSP64T vector (Krieg and Melton, 1984) and mRNA was synthesized *in vitro* as described above.

Fig. 1 is a schematic diagram of the proposed ΔE-HBDER hybrid. In the absence of hormone, the HBDER of the hybrid dominant negative mutant cadherin is occupied by the HSP90, causing steric hindrance that covers part of the β-catenin binding site present in the C-terminal part of ΔE-Cad cytoplasmic tail. The steric effect prevents the mutant protein from exerting its dominant negative effect, which involves binding to cytoplasmic β-catenin. If β-estradiol, the ligand of HBDER, is added, HSP90 detaches from the HBDER freeing the β-catenin binding domain and allowing ΔE-Cad to exert its dominant negative function.

Embryos, injections and hormone induction

Eggs were obtained from *Xenopus laevis* females injected with human chorionic gonadotrophin. The eggs were artificially fertilized and staged as described by Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Injections were performed at stage 2 or 6 N.F., depending on the experiment, with an Eppendorf microinjector. We injected 3.75 ng of ΔE-HBDER mRNA into each blastomere at stage 2 N.F. or 1.25 ng of ΔE-HBDER at stage 6 N.F.; 1 ng of β-galactosidase mRNA was coinjected as cell tracer.

The function of the protein was induced by carefully removing the vitelline membrane at different stages and adding 10 μM β-estradiol to the medium (Kolm and Sive, 1995).

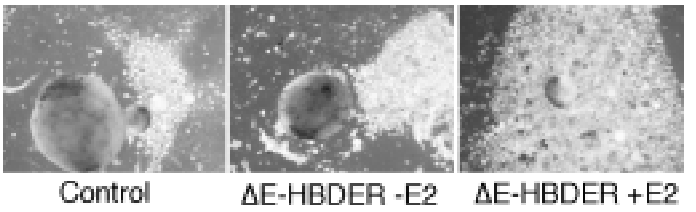

Fig. 3. The induction of ΔE-HBDER prevents cell reaggregation. *2-cell embryos were injected with mRNA encoding the inducible dominant negative cadherin. Animal caps from stage 8 N.F. embryos were disaggregated and their ability to form aggregates was tested. Cells harvested from uninjected embryos and from injected but uninduced embryos formed large aggregates, whereas cells from injected and induced embryos did not form aggregates.*

β-galactosidase staining

Embryos were fixed in 2% paraformaldehyde, 2% glutaraldehyde in PBS (phosphate-buffered saline). They were incubated overnight, with shaking, in 0.15% X-gal in PBS. Embryos were washed and dehydrated in ethanol, depigmented by incubation in 70% methanol and 10% hydrogen peroxide and rendered transparent by incubation in benzyl alcohol/benzyl benzoate (1:2).

Immunoprecipitation

Four embryos, not injected or injected with ΔE-HBDER mRNA, were pooled and incubated with 100 μl of immunoprecipitation buffer (PBS with Ca^{2+} and Mg^{2+}, 1% Triton-X-100, 1% Na-deoxycholate, 0.1% SDS, 13 μg/ml leupeptin, 2mM PMSF, 4 μg/ml aprotinin) for 30 minutes on ice. They were then centrifuged for 15 minutes at 20,000 g at 4°C. The supernatant was collected and incubated with protein-A-Sepharose (Pharmacia) for 1 hour. The mixture was centrifuged as before and the supernatant was collected and incubated overnight at 4°C with 2 μg of polyclonal pan-cadherin antibody (Takara Biomedicals, Japan). Antigen-antibody complexes were precipitated by incubation with 25 μl of protein-A-Sepharose for 2 hours at 4°C and washed four times with immunoprecipitation buffer. Proteins were eluted by boiling in Laemmli buffer. Samples were run on 8% SDS-polyacrylamide gels and the separated proteins were transferred to nitrocellulose membranes. The membranes were probed with antibodies according to standard procedures, using PBS 0.1% Tween-20 and 0.1% gelatin to block non-specific binding. The primary antibody was the same polyclonal pan-cadherin antibody used for the initial incubation, at a concentration of 1 μg/ml. The secondary antibody, a horseradish peroxidase-conjugated anti-rabbit IgG was detected by enhanced chemiluminescence.

Dissociation and reaggregation assay

Animal caps from non injected or injected stage 8 N.F. embryos were removed and transferred to agarose-coated dishes containing Ca^{2+}- and Mg^{2+}-free medium (CMFM: 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 7.5 mM Tris, pH 7.6). Animal caps were incubated for 1 hour in CMFM in the presence or absence of 10 μM estradiol. During this period, the outer layer was removed and the cells of the internal layer were dissociated. We initiated aggregation by adding $CaCl_2$ to a final concentration of 2 mM and gently rotating the dishes (Lee and Gumbiner, 1995). Aggregate formation was assessed-after two hours.

Animal cap assays

Animal caps were isolated from stage 8 N.F. embryos and incubated in 0.75 X NAM with recombinant Activin B. A dilution assay was previously performed, with dilutions from 1/2 to 1/128, to determine the concentration that generated effective elongation; a dilution of 1/50 was finally selected for use in all the tests. Statistical analysis of elongation was performed with Metamorph software (Universal Imaging).

Results and Discussion

Analysis of the expression of ΔE-HBDER construct

The expression of the inducible construct was assessed by immunoprecipitation. A pan-cadherin antibody recognizing an epitope in the cytoplasmic tail was used to detect both the native

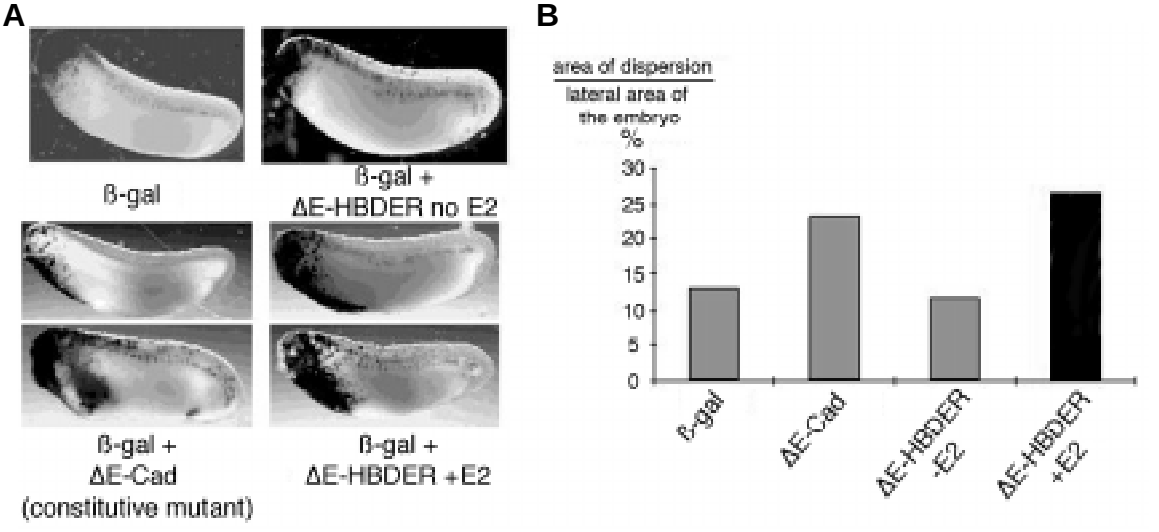

Fig. 4. The progenies of blastomeres injected with the inducible and constitutive ΔE-cadherin mutants are similarly redistributed upon induction. (A) *Stage 6 N.F. embryos were injected with mRNA encoding the constitutive or inducible dominant negative cadherin. The mRNA was injected into A1A2 blastomeres and the progeny was followed by coinjecting β-galactosidase mRNA. The progeny was similarly distributed in embryos injected with ΔE-Cad mRNA and in hormone-induced embryos injected with ΔE-HBDER mRNA. Uninduced embryos injected with ΔE-HBDER showed the same distribution as embryos injected with β-galactosidase alone.* **(B)** *The areas containing the progeny of the injected blastomeres were evaluated with Metamorph software; the ratio between the area over which the progeny were dispersed and the lateral area of the embryo is plotted.*

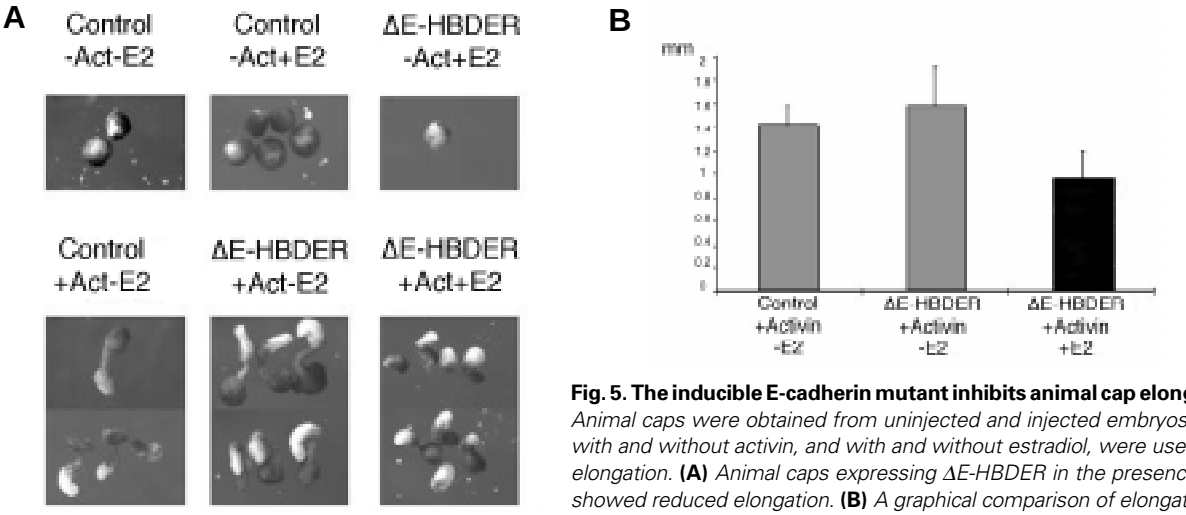

Fig. 5. The inducible E-cadherin mutant inhibits animal cap elongation upon induction. *Animal caps were obtained from uninjected and injected embryos. Various combinations, with and without activin, and with and without estradiol, were used to study the effect on elongation.* **(A)** *Animal caps expressing ΔE-HBDER in the presence of hormone induction showed reduced elongation.* **(B)** *A graphical comparison of elongation.*

and the mutant cadherin. Two bands were detected (Fig. 2): a band at 120 kDa corresponding to the endogenous cadherin and a band at 60 kDa corresponding to exogenous cadherin. The observed molecular masses are consistent with the expected values. The 60 kDa band was also detected by the anti-VSV-G antibody, which recognizes the tag inserted in the N-terminal tail of the construct (data not shown). ΔE-HBDER expression was detected as early as two hours after injection (Lanes C, D). At stage 8 N.F. the amounts of dominant negative protein endogenous cadherin were similar (Lanes F, G). No difference was observed between uninduced (Lanes C, F) and induced embryos (Lanes D, G), indicating that the conditions used for immunoprecipitation cannot be used to distinguish between the non functional and the functional state of the dominant negative mutant.

Analysis of the reaggregation capability of animal caps expressing ΔE-HBDER dominant negative mutant

Expression of truncated forms of type I cadherins during early *Xenopus laevis* development has been demonstrated to cause cell dissociation due to the loss of cell adhesion (Broders and Thiery, 1995). It has also been shown that the overexpression of a cytoplasmic dominant negative form of cadherins causes the dissociation of animal caps (Kintner, 1992; Lee and Gumbiner, 1995). We investigated whether the inducible dominant negative cadherin interfered with adhesive properties in a classical dissociation-reaggregation assay. Dissociated cells from non injected embryos stuck together in the presence of Ca^{2+}, forming large, round aggregates (Fig. 3). Similar aggregates were observed with injected but uninduced animal caps. Conversely, animal caps injected and induced with 10 μM β-estradiol did not aggregate and formed only small clusters.

Comparison of cell dispersion caused by the constitutive and inducible dominant negative mutants

It has already been shown that injecting ΔE-Cad, the constitutive dominant negative mutant, mRNA into A1-A2 blastomeres results in extensive dispersion of the derived cells at the tailbud stage (Broders and Thiery, 1995). We checked the viability of the inducible dominant negative cadherin by comparing the dispersion induced by this mutant with that induced by the constitutive ΔE-Cad. A1-A2 blastomeres are the progenitors of numerous structures including the retina, lens, brain, head mesenchyme, epidermis and bone marrow (Nakamura, 1971; Dale and Slack, 1987; Moody, 1987). We injected the cell-tracer, β-galactosidase mRNA, either alone or together with the inducible ΔE-HBDER mRNA or the constitutively active ΔE-Cad mRNA. Embryos injected with the inducible form were induced by adding β-estradiol to the medium at stage 8 N.F. and were allowed to grow to the late tailbud stage. Fixed embryos were stained for β-galactosidase and the progeny of the injected blastomeres analyzed (Fig. 4, panel A). As expected, the injection of ΔE-Cad mRNA resulted in a higher level of cell dispersion than injection of β-galactosidase mRNA alone: staining was not restricted essentially to the head but was dispersed posteriorly and ventrally. Embryos producing ΔE-HBDER, induced with β-estradiol, presented the same staining pattern as those producing ΔE-Cad, demonstrating that the induced dominant negative mutant was indeed functional. In contrast, ΔE-HBDER was not activated in the absence of the hormone, as the pattern of staining was similar to that in embryos injected with β-galactosidase mRNA alone. Thus, there was no residual activity that might complicate interpretation. We monitored the ratio between the area over which the progeny of the injected blastomeres was dispersed and the total lateral area, using Metamorph software; the data, shown in Fig. 4, panel B clearly confirm the direct observations shown in panel A.

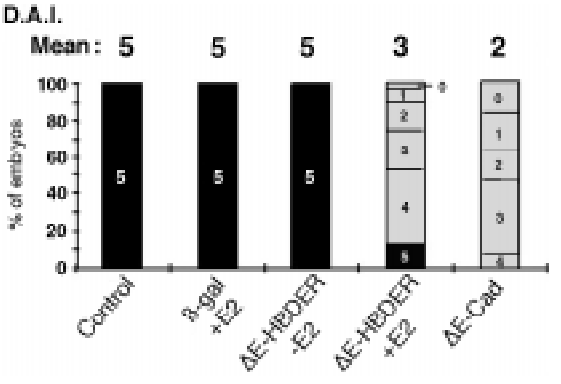

Fig. 6. β-Estradiol induced the ventralization of dorso-vegetally injected embryos. *Embryos were injected dorso-vegetally with mRNA encoding the ΔE-Cad and ΔE-HBDER mutants and were then induced. The D.A.I. are plotted in the graph, with the mean for each group of injections indicated at the top of the column.*

Analysis of the elongation of injected animal caps

The animal cap constitutively expresses EP/C-cadherin and then, from the beginning of gastrulation, E-cadherin. If cultured alone, this explant forms a spherical mass of tissue. If cultured in the presence of activin, a mesoderm-inducing factor belonging to the TGF-β family, it undergoes dramatic elongation, due to morphogenetic movements related to gastrulation. Several studies using either antibodies (Brieher and Gumbiner, 1994) or dominant negative forms (Broders and Thiery, 1995) that interfere with cadherin function, have shown that inhibition of the elongation of induced animal caps is related to the loss of function of type I cadherins. We carried out a similar analysis with the inducible ΔE-Cad and found that the inhibition of elongation was hormone-dependent. Results are shown in Fig. 5 panel A and B. Animal caps not induced with β-estradiol behaved like uninjected animal caps and β-estradiol alone did not interfere with the elongation process. This simple test confirmed that the inducible form had a similar effect to the constitutive dominant negative form only if hormone was added.

Comparison of the ventralization index of injected embryos

Numerous studies have demonstrated that β-catenin is necessary for establishment of the dorsoventral axis. The injection of β-catenin mRNA into the ventral side of embryos induces secondary axis formation (Funayama *et al*., 1995) whereas the injection of antisense oligonucleotides leads to ventralized embryos (Heasman *et al*., 1994). Type I cadherins, which normally bind to β-catenin, have been shown to modulate the dorsalizing function of this molecule: the overproduction of C-cadherin in dorso-vegetally injected embryos leads to the loss of dorsal structures (Fagotto *et al*., 1996). It has been shown in our laboratory, in terms of both biochemistry and function, that the intracellular dominant negative mutant exerts its function by recruiting β-catenin (Broders and Thiery, 1995). We investigated whether ΔE-HBDER caused the same phenotype as ΔE-cad due to recruitment of the cytoplasmic pool of β-catenin. We injected 2-cell stage embryos dorso-vegetally to target mRNAs to the area close to the Nieuwkoop center. Embryos were induced at stage 8 N.F. and the dorso-anterior index was analyzed as described by Kao and Elinson (Kao and Elinson, 1988). The results are shown in the graph of Fig. 6. Embryos injected with either β-galactosidase mRNA or ΔE-HBDER mRNA but not induced had a mean D.A.I. of 5, whereas embryos injected with ΔE-Cad mRNA and those injected with ΔE-HBDER mRNA and induced had a much lower mean D.A.I., indicating strong ventralization. This experiment confirms that the inducible form acts in a similar way to the constitutive form, but only under hormone induction. Furthermore, it shows that the dominant negative effect of the inducible mutant, like that of the constitutive mutant, depends on the recruitment of cytoplasmic β-catenin.

Conclusions

In this study, we generated a fusion protein comprising the dominant negative ΔE-Cad and the hormone-binding domain of the estradiol receptor, HBDER, to obtain a hormone-inducible function with time-specific switching.

We first checked the expression of the dominant negative chimera by immunoprecipitation and showed that the protein was present in physiologically significant amounts from very early stages onwards. Unfortunately, we were unable to determine whether the protein was into a functional or non functional state by biochemical means so, as described in previous studies (Kolm and Sive, 1995), we established that 1 hour (see the aggregation assay) of 10 μM β-estradiol induction was sufficient to convert the chimera to the active state.

We then checked that the inducible dominant negative cadherin was functional. In all the assays performed in this work the hybrid protein, when induced by hormone, behaved similarly to the ΔE-Cad constitutive mutant. The effects observed were: (1) abolition of cell aggregation, (2) cell dispersion, (3) inhibition of elongation induced by the activin of the animal caps and (4) dissociation of the animal pole (data not shown). The hybrid mutant had no effect in the absence of hormone induction. Thus, this hormone-inducible system is suitable for production of the dominant negative mutant and, combined with targeted injections at stage 6 N.F., provides a powerful new tool for investigating the role of type I cadherins at various post-gastrulation developmental stages.

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