Depression in the Level of Cadherin and α-, ß-, γ-catenins in Transgenic *Xenopus laevis* Highly Expressing c-Src

(c-Src / cadherin / α-, ß-, γ-(plakoglobin) catenins / RSV LTR / *Xenopus laevis*)

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Abstract. Aberrant morphogenesis of transgenic *Xenopus laevis* 5-day embryos carrying Rous sarcoma virus LTR in their DNA and expressing a high level of c-Src protein kinase was found to be accompanied with a profound depression in the level of cadherins and α-, ß-, and γ-(plakoglobin) catenins in their tissues, as revealed by immunohistochemical analysis. Simultaneously, an increased level of phosphotyrosine staining was detected. However, an analogous increase in the level of phosphotyrosine immunostaining and a slightly higher level of Src were also detected in tissues of originally defective but later spontaneously repaired frog embryos that displayed essentially normal patterns of staining for cadherins and catenins. Our results provide evidence that the defective morphogenesis of frog embryos expressing a high level of c-Src is characterized by the loss of the cadherin-catenin complexes. It appears that to induce frog morphogenetic malformations, the c-Src overproduction and the loss of cadherins-catenins are simultaneously required. Phosphorylation is not likely to be the cause of cadherin and catenin disappearance from the tissues of strongly aberrant frog embryos.

The cytoskeletal-membrane association at cell-cell contacts plays a central role in determining the cell shape, tissue integrity or cell sorting that are necessary to position different cell types during development (Broders and Thiery, 1995). It has become clear in recent years that some proteins at contact points called adherens junctions and desmosomes display not only properties of linker molecules, but have functions in signal transduction pathways and regulation of gene expression. Both adhesion and signaling functions have profound effects on the cell behavior during development (Cowin and Burke, 1996).

Received September 21, 1999. Accepted November 9, 1999.

This work was supported by the grant 312/96/K205 from the Grant Agency of the Czech Republic.

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Abbreviations: GSK – glycogen synthase kinase 3ß, LEF – lymphoid enhancer binding factor, LTR – long terminal repeat, P-Tyr – phosphotyrosine, RSV – Rous sarcoma virus, Src – protein tyrosine kinase $pp60^{src}$ (the product of the *src* gene), TCF – T-cell-specific factor.

Cell-cell contacts at the adherens junctions are mediated by Ca^{2+}-dependent transmembrane glycoproteins cadherins (such as E-cadherin, P-, B-, L-CAM-, or N-cadherin). The cadherin function is controlled by cytoplasmic proteins catenins associated with the highly conserved cytoplasmic domain of cadherins. ß- and γ-catenin/plakoglobin, which are homologous, bind to the intracellular domain of the cadherin tail directly. α-catenin interacts with sites shared by ß- and γ-catenin linking the ß- or γ-catenin/plakoglobin/cadherin complex to the actin cytoskeleton (see Finnemann et al., 1997, for references). An additional catenin family member, the protein $p120^{ctn}$, binds directly to another cytoplasmic part of cadherin, but does not interact with α-catenin (Daniel and Reynolds, 1997). A recent report from the same laboratory (Daniel and Reynolds, 1999) describe3s a unique binding partner of $p120^{ctn}$, a novel transcription factor named Kaiso, suggesting a distinct role for $p120^{ctn}$ in the cadherin-catenin system.

$p120^{ctn}$, ß-catenin and γ-catenin/plakoglobin are structurally similar. They are called 'Arm catenins', because each is containing a central armadillo repeat domain (Arm domain). By sharing common mediator proteins, ß-catenin and γ-catenin/plakoglobin, the cadherin adhesion system is coupled to the signaling pathways activated by molecules of the *wnt* family, which represent the vertebrate homologues of the segment polarity gene *wingless* in *Drosophila* affecting embryonal axis specification (Peifer and Wieschaus, 1990; Gumbiner, 1995; Karnovsky and Klymkowsky, 1995; Larabell et al., 1997; Moon et al., 1997). The failure either to assemble the cadherin-catenin complex or to properly connect to the actin cytoskeleton results in the loss of cell adhesion (Semb and Christofori, 1998). The role of cadherins in histogenesis, the assembly of complex tissues during embryonic development, has been extensively studied and recognized also in *Xenopus* embryos (Kuhl and Wedlich, 1996; Bradley et al., 1998). Ectopic overexpression of ß-catenin in *Xenopus* was described to induce the formation of a complete secondary body axis (Funayama et al., 1995).

Several Src family kinases have been indirectly implicated in the cadherin function. c-Src, c-Yes, and c-Fyn are enriched at adherens junctions (Tsukita et al. 1991),

which are the major sites of tyrosine-specific protein phosphorylation (Volberg et al. 1992), especially during development (Maher and Pasquale, 1988; Takata and Singer, 1988). Inhibition of tyrosine phosphatases leads to a rapid increase in adherens junction-associated phosphotyrosine and to disassembly of junctions. Conversely, in Src-transformed cells, adherens junctions disrupted by transformation are reformed after treatment with tyrosine kinase inhibitors. Studies with MDCK cells expressing temperature-sensitive Src mutants suggest that tyrosine phosphorylation/Src molecules directly alter cell morphology (Volberg et al., 1992). N-cadherin and catenins of chicken embryonic fibroblasts were found to be very good substrates of v-Src, and their phosphorylation on tyrosine correlated with suppression of cell-cell adhesion (Hamaguchi et al., 1993; see also Behrens et al. 1993). The 'Arm catenins' were reported to be the major protein tyrosine kinase (PTK) substrates in cadherin complexes, and are therefore likely to account for most of the PTK-induced effects on cadherin function (Daniel and Reynolds, 1997). Thus, through regulation of the assembly and dissociation of the cadherin-cytoskeleton complex, tyrosine kinases may regulate the cell shape, migration, proliferation and tissue integrity (Cowin and Burke, 1996), and via ß-catenin join the Wnt signaling pathway (Hinck et al., 1994). Several growth factors including fibroblast growth factor 1, epidermal growth factor, scatter factor/hepatocyte growth factor, platelet-derived growth factor and colony stimulating factor 1 were shown to induce activation of c-Src, resulting in cytoskeletal remodeling apparently due to enhanced tyrosine phosphorylation of ß- or γ-catenins. In turn, Src can also bind, phosphorylate and activate receptors of some of the growth factors (Belsches et al., 1997; Thiery and Chopin, 1999). Both Src and Ras were reported to control the epidermal growth factor- and hepatocyte growth factor-induced scattering of NBT-II carcinoma epithelial cells, but to operate in separate pathways (Boyer et al., 1997; Thiery and Chopin, 1999).

In our laboratory, plasmid pAPrC (Meric and Spahr, 1986) carrying the complete proviral form of the Rous sarcoma virus (RSV) genome was repeatedly transferred into eggs using the sperm of *Xenopus laevis*, giving rise to transgenic frogs of F_0 generation (Jonák et al., 1994; Habrová et al., 1996). By mating a fertile F_0 transgenic male with a normal female, several B_1 generations of transgenic frog embryos were prepared carrying in their genome some portions of RSV DNA, namely long terminal repeat (LTR) sequences, comprising strong promoters and enhancers, and most of the *pol* gene (Takáč et al., 1998). Some B_1 transgenic embryos (15-20%) developed various morphogenetic malformations like edemas, head deformities, and eye and axial system defects. The occurrence of these aberrations was accompanied with high expression of c-Src kinase in their tissues; a small increase in c-Src expression was compatible with normal embryogenesis or with spontaneous reversion of mild morphogenetic defects and transition into apparently normal development. The expression of c-Src correlated with the presence of RSV LTR integrated in frog DNA. These findings led to the hypothesis that the integrated RSV LTR could stimulate the expression of the *c-src* proto-oncogene(s) of *X. laevis* embryos, and when the concentration of the c-Src protein exceeds a certain threshold, it interferes with their early developmental program (Takáč et al., 1998).

In the present work the correlation between the high c-Src expression and aberrant morphogenesis was further investigated from the point of view of the above described cell-cell adhesion mechanisms. The dosage of the Src kinase in tissues of transgenic frog embryos was compared with the dosage of individual components of the cadherin-catenin system by immunohistochemical analysis. The results show a clear correlation between a high Src level and a profound depression in the level of cadherin and catenins in strongly defective frog embryos. Staining for phosphotyrosines also differred between both groups of organisms.

Material and Methods

Antibodies

Primary antibodies anti-pan cadherin (C 3678), anti-catenin α (C 2081), anti-catenin ß (2206), anti-plakoglobin (anti-catenin γ, P8087) and anti-phosphotyrosine (P 3300) were purchased from Sigma-Aldrich, Prague, Czech Republic. Anti-Src (v-*src*, Ab-1), recognizing both c-Src and v-Src, was from Oncogene Science, Inc., Uniondale, NY. Secondary antibodies SwAR/FITC and SwAM/FITC were purchased from Sevac, Prague, Czech Republic. All antibodies were used according to the manufacturers' recommendations.

Frog embryos

Preparation of B_1 generation of transgenic *Xenopus laevis* was described previously (Takáč et al., 1998). Defective embryos were divided into mildly defective and strongly defective. The mildly defective embryos were characterized by retarded development, edemas and spontaneous reparation in most cases. The strongly defective embryos were characterized by head deformities, eye defects, axial defects, and no reparation. The ratio between the strongly defective and the mildly defective 5-day B_1 embryos was about 26 : 43 in a typical experiment (see details in Table 1, Takáč et al., 1998).

Indirect fluorescence

Longitudinal sections of 5-6 μm width through embryo bodies were mounted and prepared as described in Habrová et al. (1996) with small modifications. Incubation with a primary antibody took place at room temperature overnight, and after repeated washing with 130 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4,

pH 7.4 buffer, the incubation with a secondary antibody labeled with fluorescein took place for at least 2 h. Sections were examined under a fluorescence microscope Olympus BX-40 equipped with a U-M51 006 filter cube and photographed using an automatic Olympus PM-20 camera (Olympus, Tokyo, Japan) and KODAK Ektapress GoldII Professional ISO 400 film (Eastman Kodak Corp., Rochester, NY). Positive response: green fluorescence.

Results

The primary antibodies against adhesion proteins applied here were either of rabbit or mouse origin, and it was necessary to verify their specificity against cadherin-catenin components of frog organisms. For this purpose western-blot analyses of homogenates from 5-, 14-, 21- and 60-day intact *X. laevis* were carried out. It was proven that all antibodies reacted on western blots specifically only with proteins of molecular masses corresponding to individual adhesion proteins of the cadherin-catenin system of the frog (results not shown). The staining with the anti-catenin α antibody gave generally weaker signals than that with the anti-pan cadherin, anti-catenin ß or anti-catenin γ antibodies.

Development of frog embryos proceeds very rapidly, and at the age of 5 days the differentiation into individual tissues is well observable at the microscopic level. As shown in Fig. 1A, staining of tissue sections of 5 day-control, intact embryos for the Src kinase protein with a specific Ab-1 monoclonal antibody was almost negligible throughout the whole organism. Very low immunofluorescence was detectable only in the neuronal system. The level of phosphotyrosine labeling was also low. The staining was poorly detectable in the nerve cord and somites, and it was a little more positive in myoblasts and in the caudal part of the chorda (Fig. 1B). On the other hand, staining for cadherins with anti-pan cadherin antibody recognizing all types of cadherins (Fig. 1C), as well as for ß-catenin (Fig. 1E) and for γ-catenin/plakoglobin (Fig. 1F) with respective antibodies, was very well pronounced in all tissues of intact embryos. The staining for α-catenin was less intensive than for cadherins, but clearly detectable in all tissues, e. g. in the spinal cord, brain, somites, notochord, pharynx, otic vesicles, midgut, succers, pronephrotic tubules, aorta, as well as basal membrane (Fig. 1D, and results not shown).

Immunohistochemical examination of individual tissues of strongly defective transgenic *X. laevis* embryos was more complicated. This was mainly due to profound tissue disorganization, in many cases preventing its unambiguous identification, and to an overall delay in the developmental process in aberrant embryos. The lack of correct foundation of brain, eye and ear tissues was a general phenomenon. Most interestingly, the immunostaining pattern was principally different in strongly defective B_1 transgenic *X. laevis* embryos from that of intact embryos. As described previously (Takáč et al., 1998) and confirmed here, Src fluorescence was very high in all examined tissues of the defective embryos, e.g. in the nerve cord, notochord, and somites (Fig. 2A). The high Src expression was followed with an increased fluorescence of phosphotyrosines (Fig. 2B). The phosphotyrosine (P-Tyr) staining was detectable e.g. in the nerve cord, myoblasts, otic vesicles, basal membrane, digestion and excretion system. Immunostaining for cadherins (Fig. 2C), α-catenin (Fig. 2D) and γ-catenin (Fig. 2F) gave completely negative results. Immunodetection of ß-catenin (Fig. 2E) was also unsuccessful, except in the dorsal part of the brain where a few positive signals were obtained. This essentially complete absence of all components of the cadherin-catenin system in the strongly aberrant frog embryos was not expected. The phenomenon of a simultaneous downregulation of cadherins and catenins has not been reported before. The lack of immunostaining might be theoretically also due to a distorted epitope structure of the target proteins, but it is apparently not the case here. It is difficult to imagine that the distortion would take place in all four examined types of proteins of the cadherin-catenin complex and at the same time.

The complete absence of cadherins and catenins was typical only for strongly defective embryos. Immunostaining for these proteins in mildly defective and later spontaneously repaired embryos provided patterns and intensities that were very similar to or indistinguishable from those of control embryos. As an example, ß-catenin staining patterns of an eye section from a control embryo (Fig. 2G) and a defective-repaired embryo (Fig. 2H) were compared. This suggests that a defect in the expression of these adhesion proteins is the primary cause of the aberrant development and differentiation arrest. When the expression of the adhesion proteins was resumed and the expression block was overcome, the development of the organisms continued further, irrespective of the presence of RSV LTR sequence in their genome and a slightly increased Src expression (see Takáč et al. 1998).

Discussion

Regulation of the synthesis of individual proteins of the cadherin-catenin complex has not been elucidated yet. The coincidence between the high c-Src expression and the disappearance of these proteins from tissues observed in our experiments might suggest an involvement of the kinase in the cadherin-catenin regulatory pathways. High c-Src might block their synthesis and/or stimulate rapid degradation of cadherin-catenin complexes. ß-catenin degradation is regulated by a component of the Wnt signaling pathway, glycogen synthase kinase 3ß (GSK). GSK phosphorylates ß-catenin on specific Ser and Thr residues and prepares it for degradation by the ubiquitin-proteasome system (Yost et al., 1996).