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Requirement of the juxtamembrane domain of the cadherin cytoplasmic tail for morphogenetic cell rearrangement during myotome development

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During development, the activity of cadherin cell adhesion molecules is assumed to be regulated to allow for cell rearrangement or translocation. Previous studies suggest that the juxtamembrane (JM) domain of the cadherin cytoplasmic tail, which contains the site for binding to p120^{ctn}, has a regulatory function in this adhesion system. To study the possible role of JM domain–dependent cadherin regulation in embryonic cell rearrangement, we ectopically expressed a series of N-cadherin mutants in developing somites of chicken embryos. When a JM domain–deficient N-cadherin was expressed, the morphogenetic expansion of the myotome was strongly suppressed. However, a triple alanine substitution in the JM domain, which specifically inhibited the p120^{ctn} binding, had no effect on myotome development. Furthermore, a dominant negative N-cadherin, which had a deletion at the extracellular domain but maintained the normal cytoplasmic tail, did not affect myotome expansion; although it disrupted intersomite boundaries. Overexpression of p120^{ctn} also did not affect myotome expansion, but it did perturb myofiber orientation. These and other observations suggest that the JM domain of N-cadherin has a regulatory role in myotome cell rearrangement in which molecules other than p120^{ctn} are involved. The p120^{ctn} molecule itself seems to play a critical role in the arrangement of myofibers.

Introduction

Cell rearrangement is an essential step for animal morphogenesis. In many developmental processes, cells dynamically change their positions, neighbors, shape, and arrangement pattern; and some of such phenomena are called convergent extension or cell intercalation. Examples requiring cell rearrangement in early vertebrate development include gastrulation, notochord elongation, neural crest emigration, and somite differentiation. For these processes to proceed normally, cell–cell adhesion must be regulated; otherwise, cells would not be able to change their neighbors or to move. Therefore, to understand how cell–cell adhesion is regulated is an important issue for elucidating cell rearrangement mechanisms.

The cadherin adhesion system plays a key role for the development of tight association between cells. However, this system is also known to be required for some cell rearrangement processes (Brieher and Gumbiner, 1994; Lee and Gumbiner, 1995; Uemura et al., 1996) and even for cell movement (Oda et al., 1997; Niewiadomska et al., 1999). These findings suggest the presence of mechanisms that allow reversible cadherin-mediated cell adhesion. In fact, the cytoplasmic proteins associated with the cadherin cytoplasmic tail, collectively called catenins, are believed to regulate cadherin activities (for review see Gumbiner, 2000). The cytoplasmic tail is roughly divided into two portions, the NH_2-terminal half (juxtamembrane [JM]*) domain and the remaining COOH-terminal half domain to which p120^{ctn} and β-catenin, respectively, are known to bind (for review see Barth et al., 1997). Previous studies demonstrated that the JM domain of the cadherin cytoplasmic tail plays an important regulatory role in cadherin function (Aono et al., 1999): colon carcinoma Colo205 cells could not establish cadherin-dependent compact aggregates even though they expressed a normal set of molecules required for the cadherin adhesion machinery, that is, E-cadherin, α- and β-catenin,

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*Abbreviations used in this paper: DV, dorsomedial-ventrolateral; JM, juxtamembrane; MHC, myosin heavy chain.

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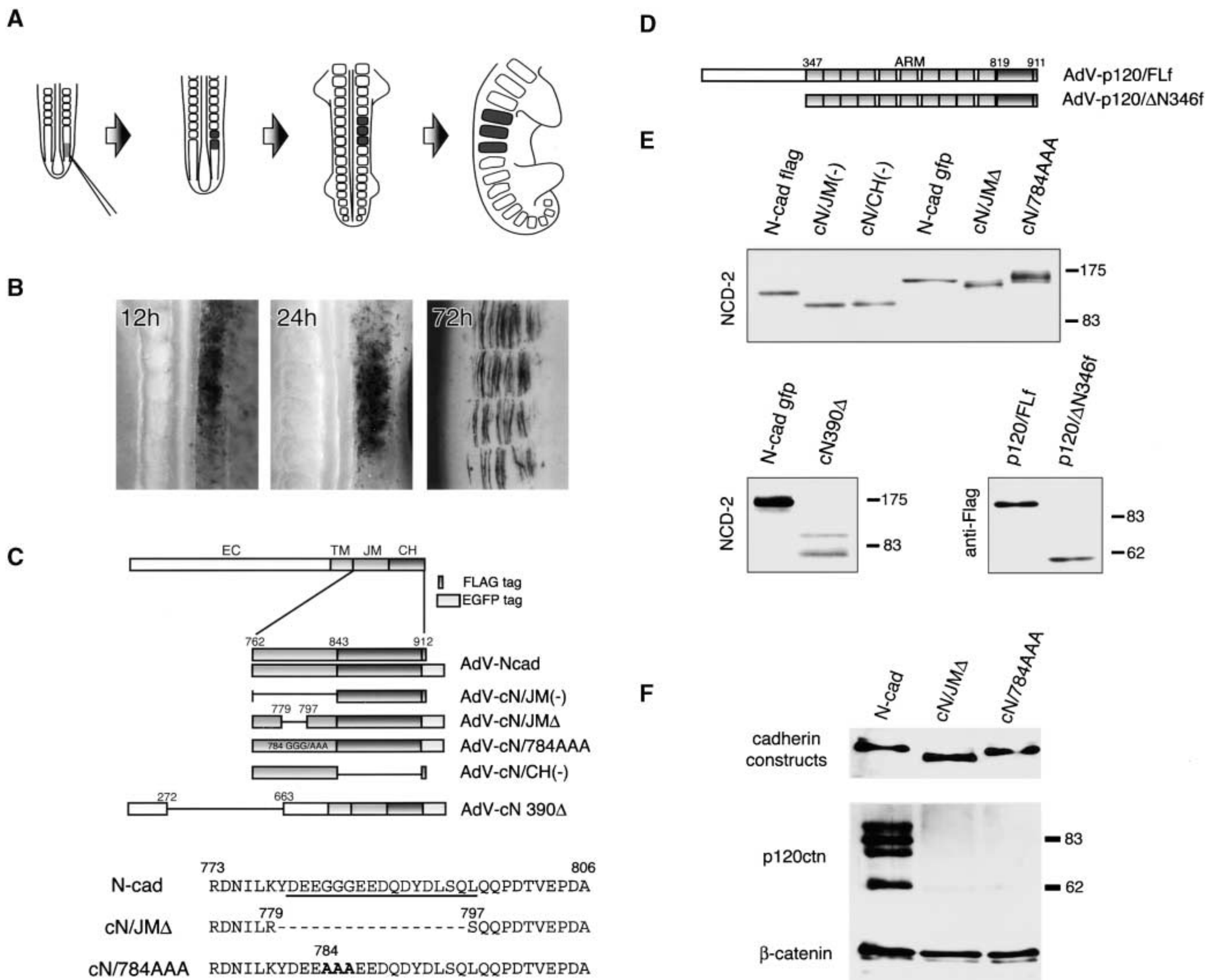

Figure 1. **Schematic illustration of the experimental design and basic information.** (A) Adenoviral expression vectors were injected into the segmental plate of stage 12 embryos. The earliest protein expression begins at 6–8 h after injection and persists at least for 3 d as depicted by the shadowing. (B) Whole-mount X-gal staining of embryos injected with adenoviral vectors encoding *lacZ* (AdV-lacZ) at 12, 24, and 72 h after the injection. Although the transgene is expressed in the entire segmenting mesoderm at early stages, a high level of gene expression is maintained only in myofibers at 72 h. (C and D) Wild-type and mutated N-cadherins (C) or $p120^{ctn}$ (D) for constructing recombinant adenoviruses. Each construct is tagged by EGFP or FLAG epitope at their COOH terminus. Amino acid sequences at the mutated portion in cN/JMΔ and cN/784AAA are shown. The underlined region was suggested to be crucial for binding to $p120^{ctn}$ (Thoreson et al., 2000). EC, extracellular domain; TM, transmembrane domain; JM, juxtamembrane domain; CH, carboxy half domain; ARM, armadillo repeat. (E) Western blots of the recombinant N-cadherins and $p120^{ctn}$ were studied. Cadherin-deficient A431D cells were infected with respective viral vectors, and expressed proteins were detected with the monoclonal antibody NCD-2, which recognized the NH_2-terminal region of N-cadherin. For examination of cN390Δ, the original A431 line was used for infection because this construct was unstable in cadherin-deficient cells. The immunoreactivity of cN390Δ to NCD-2 was reduced compared with that of the full-length N-cadherin (Fujimori and Takeichi, 1993), resulting in an apparently faint band. The higher MW band in the cN390Δ lane is presumably a precursor protein. $p120^{ctn}$ was detected with anti-Flag antibodies. Viral vectors of the same titer as used for these infections were used for in ovo infection. (F) Western blot detection of $p120^{ctn}$ from the immunoprecipitants of N-cad, cN/JMΔ, and cN/784AAA. These proteins were expressed in A431D cells as above, immunoprecipitated with rabbit anti–N-cadherin antibodies, and subjected to detection of $p120^{ctn}$. This protein was not detected in the cN/JMΔ and cN/784AAA immunoprecipitates. Multiple $p120^{ctn}$ bands coprecipitated with the control N-cadherin represent isoforms of this protein (Anastasiadis and Reynolds, 2000). As a control, β-catenin was detected from all the samples.

and $p120^{ctn}$. However, in these cells a normal cadherin activity could be restored by transfecting them with a mutant cadherin lacking the JM domain or with NH_2 terminus–deleted mutants of 120^{ctn}. From these observations, we hypothesized that the JM domain has an inhibitory role in cadherin function in the Colo205 cells and that $p120^{ctn}$ is involved in the regulation of this inhibition. The idea to assign the JM domain or $p120^{ctn}$ to regulate cadherin function was supported by the results of other studies (Ozawa and Kemler, 1998; Yap et al., 1998; Thoreson et al., 2000). Involvement of the JM domain in cell motility was also suggested (Riehl et al., 1996; Chen et al., 1997). Furthermore, the JM domain was implicated in regulation of integrin function (Lilien et al., 1999; Arregui et al., 2000). Recent

studies showed that p120[ctn] overexpression affected the activities of small GTPases (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001).

For the present study, we designed experiments to test whether the JM domain plays any role in morphogenetic rearrangement of embryonic cells in vivo, focusing on somitogenesis. Somites are initially epithelial spheres, and then they differentiate into two portions, sclerotome and dermomyotome. The latter cell sheet expands gradually along the dorsomedial-ventrolateral (DV) axis, producing myotome cells at each of the dorsomedial and ventrolateral lips; these cells move beneath the dermomyotome or dermatome to form the myotome layer (Denetclaw et al., 1997; Kalcheim et al., 1999; Denetclaw and Ordahl, 2000). Then, the myotome cells elongate along the rostrocaudal axis of the body, differentiating into myofibers that are arranged in parallel to each other within each somite unit. It is likely that these processes require the precise control of cell movement and rearrangement. To test the role of N-cadherin and its JM domain in these processes, we injected the full-length N-cadherin or its mutant molecules, and also p120[ctn], into developing somites and observed the effect of their ectopic expression on somitogenesis. Our results indicate that JM domain–dependent regulation of cadherin function is required for the proper arrangement of myotome cells during somite development.

Results

Expression of N-cadherin without the JM domain blocks myotome expansion

As a preliminary experiment, we injected adenoviral expression vectors encoding *lacZ* (AdV-lacZ) into the segmental plate of embryos at stage 12 (Fig. 1 A) and stained the enzymic products to determine the general expression profile of the cDNAs injected into this site. X-gal staining showed that the injected cDNA began to be expressed at ~6–8 h after the injection (unpublished data). Initially, the entire population of somitic cells were stained, and then gradually the intense staining became restricted to the myotome, resulting in visualization of only an array of myofibers in each somite (Fig. 1 B). This result can be explained based on the following two facts: (a) adenoviral vectors do not replicate, and thus they become diluted in rapidly proliferating cells and (b) myotome cells enter into the postmitotic state at early developmental stages (Kahane et al., 1998). Therefore, myotome cells would be expected to maintain a high level expression of the injected cDNAs. Because of these observations, the following experiments were focused on the myotome, which likely responds most effectively to the ectopically expressed molecules.

We constructed adenoviral expression vectors for a series of N-cadherin mutants (Fig. 1 C) whose protein products, expressed in cell lines, were shown in Fig. 1 E. We first injected the one encoding the full-length N-cadherin (AdV-Ncad) or a mutant N-cadherin without the JM domain (AdV-cN/JM[−]) into the segmental plate together with AdV-lacZ. Whole-mount X-gal staining of these embryos, incubated for 3 d after injection, showed that in AdV-Ncad–injected embryos LacZ-positive myofibers became distributed normally, whereas in AdV-cN/JM(−)–injected embryos the stain signals clustered abnormally at a mid-lateral position of the trunk (Fig. 2, A–D), indicating that the DV myotome expansion was prohibited. Then, we examined sections of such treated embryos that had been doubly stained for the tag attached to the cadherin constructs and for β-catenin (Fig. 2, E and F). Tag-positive cells in AdV-Ncad–injected embryos were distributed in a pattern characteristic of the normal myotome. In contrast, in AdV-cN/JM(−)–injected embryos tag-labeled cells were clumped in a region at the level of motor axon exit, consistent with the above whole-mount observations. The staining for β-catenin was used to visualize the overall tissue architecture, and by it most tissues appeared normal with the exception of the myotome in the AdV-cN/JM(−)–injected embryos.

Next, we observed different stages of development to determine when the abnormal somite morphogenesis began in AdV-cN/JM(−)–injected embryos. As seen in Fig. 3, E–H,

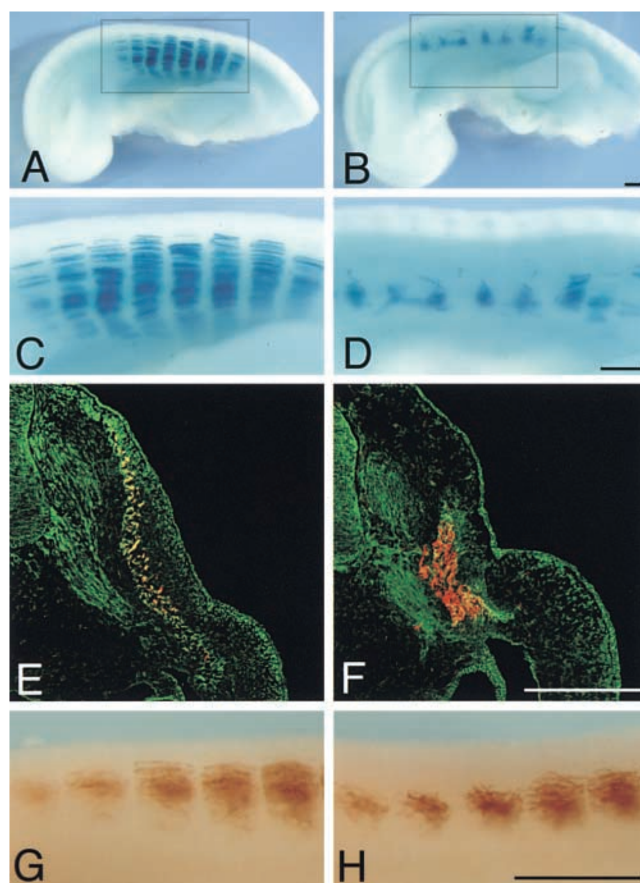

Figure 2. **Expression of the JM domain–deficient cadherin affects the morphology of myotomes.** Embryos were coinjected with AdV-lacZ and AdV-Ncad (A and C) or AdV-cN/JM(−) (B and D) and subjected to whole-mount X-gal staining at 72 h after the injection. Boxed regions in A and B are enlarged in C and D, respectively. Embryos were oriented with their rostral aspect to the right. (E and F) Transverse sections of embryos injected with AdV-Ncad (E) or AdV-cN/JM(−) (F) were double immunostained for the tag-bearing cadherin (red) and β-catenin (green) at 72 h after the injection. Myotome cells expressing the JM(−) cadherin are clumped in a region at the level of motor axon exit. (G and H) Embryos injected with AdV-Ncad (G) or AdV-cN/JM(−) (H) were immunostained as whole-mount samples for a muscle differentiation marker, MHC, at 36 h after the injection. Expression of neither control nor JM(−) cadherin did not affect muscle differentiation. Bars, 500 μm.

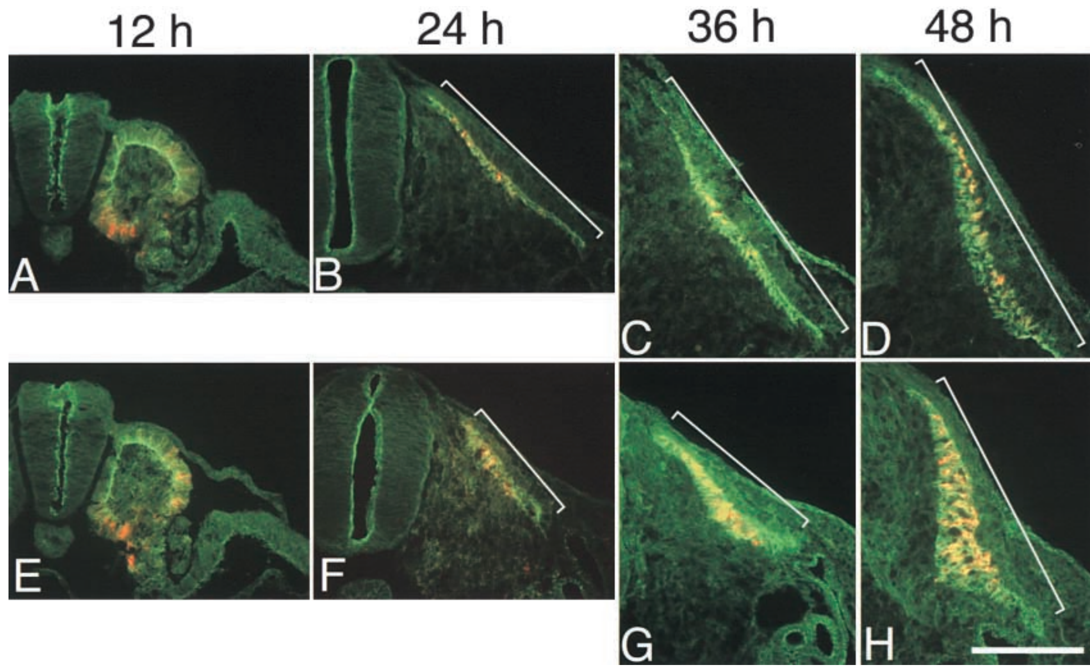

Figure 3. **Observations on the developmental effect of the JM(−) cadherin expression.** Embryos were injected with AdV-Ncad (A–D) or AdV-cN/JM(−) (E–H) and double stained for the cadherin-attached tag (green) and β-catenin (red) at 12, 24, 36, and 48 h after the injection. Expression of the JM(−) cadherin did not affect the organization of epithelial somites (E). However, the expansion of the myotome along the DV axis was inhibited from the beginning of this process. Bar, 250 μm.

at the epithelial stage somites expressing the JM domain–deficient (JM[−]) cadherin appeared to be normal, whereas as soon as myotome differentiation began its DV expansion was retarded. To further analyze the abnormal patterning of myotome in AdV-cN/JM(−)–injected embryos, we coinjected the AdV-cN/JM(−) with a relatively low titer of AdV-lacZ. This procedure allowed us to label selectively a subpopulation of myotome cells with LacZ that enter into the postmitotic phase at earlier developmental stages, since this marker is diluted considerably in cells which continue to proliferate until later stages. In embryos expressing the normal N-cadherin, most LacZ-positive cells were localized adjacent to the dermatome (Fig. 4 A), following the normal sequence of myotome cell translocation (Denetclaw et al., 1997; Kahane et al., 1998; Denetclaw and Ordahl, 2000). However, in AdV-cN/JM(−)–injected embryos, LacZ-posi-

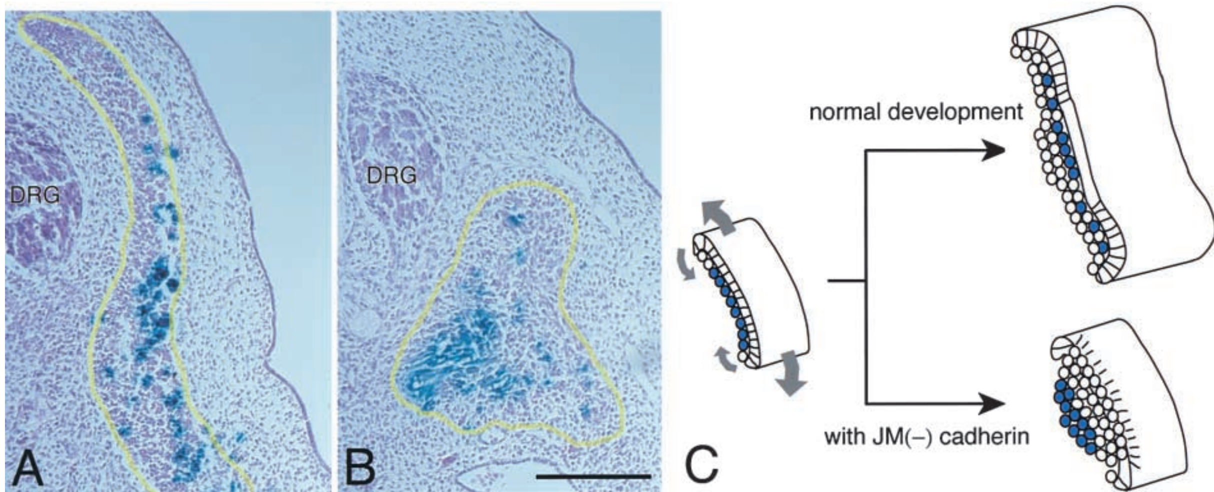

Figure 4. **JM(−) cadherin effects on the relative positioning of early and late differentiating myofibers.** Transverse sections of embryos coinjected with a smaller amount of AdV-lacZ and a normal level of AdV-Ncad (A) or AdV-cN/JM(−) (B) were subjected to X-gal staining at 96 h after the injection. In this injection strategy, only early differentiating myotome cells retain X-gal reactivity because the enzyme is diluted by excessive proliferation in late differentiating myotome cells. In the control situation, myofibers that had differentiated at earlier stages have positioned themselves at the outer side of the myotome (A), and this pattern was reversed in AdV-cN/JM(−)–injected embryos (B). Yellow outlines mark myotome cell clusters. (C) Schematic illustration of myotome cell arrangement with or without the JM domain–deficient cadherin. Bar, 500 μm.

tive cells accumulated at a deeper position within the myotome cluster with LacZ-negative cells localizing at the surface side (Fig. 4 B). Thus, the ordered myotomal cell colonization pattern was reversed in AdV-cN/JM(−)–injected embryos as summarized in Fig. 4 C, indicating that the effect of AdV-cN/JM(−) expression involved a disturbance of cell positioning.

Then, we asked whether the JM(−) cadherin expression affected muscle differentiation. Immunostaining for myosin heavy chain (MHC) showed that this muscle differentiation marker was normally expressed in myotome cell clumps induced by AdV-cN/JM(−) injection and in myotomes of control AdV-Ncad–injected embryos (Fig. 2, G and H), indicating that muscle differentiation was not affected by these treatments.

Effects of expression of other N-cadherin mutants and p120^{ctn} on myotome development

The JM domain is known to have the site to which p120^{ctn} binds, that is, the JM(−) cadherin cannot bind to this catenin (Yap et al., 1998; Ohkubo and Ozawa, 1999; Thoreson et al., 2000). To examine if the loss of p120^{ctn} was essential for the above biological action of the JM(−) cadherin, we constructed recombinant adenoviruses encoding mutant N-cadherins, cN/784AAA, and cN/JMΔ in which a triplet AAA was substituted for GGG at the putative p120^{ctn} binding site in the JM domain (AdV-cN/784AAA), identified for E-cadherin (Thoreson et al., 2000), and a region of 17 amino acids covering the p120^{ctn} binding site was deleted (AdV-cN/JMΔ), respectively (Fig. 1 C). Immunoprecipitation experiments confirmed that both of these constructs were unable to bind to p120^{ctn} (Fig. 1 F).

Injection of AdV-cN/784AAA had no effect on myotome formation; whereas AdV-cN/JMΔ showed the same effect as AdV-cN/JM(−) (Fig. 5, A–D), thus indicating that the loss of the ability of cadherin to bind to p120^{ctn} was not essential for the above action of the JM(−) cadherin and that the cadherin activity to affect myotome expansion must be ascribed to some other mechanisms associated with the above 17 amino acid region.

We also injected p120^{ctn} itself (AdV-p120/FLf encoding the p120^{ctn} isoform 1N [Anastasiadis and Reynolds, 2000]) and its NH_2 terminus deletion mutant, ΔNp120^{ctn} (AdV-p120/ΔN346f), which is known to activate the cadherin system in Colo205 cells (Aono et al., 1999). Expression of these molecules had no effect on myotome expansion (Fig. 5, I–L). However, the overexpression of the full-length p120^{ctn} exhibited a drastic effect on another phenomenon; that is, it randomized the orientation of myofibers, leading some cells to elongate even toward the dorso-ventral direction (Fig. 5, I and J). ΔNp120^{ctn} had no such effect, indicating that the above action of p120^{ctn} required the NH_2-terminal domain. Thus, p120^{ctn} seems to be involved in the regulation of the rostrocaudal extension of myofibers but not in the DV expansion of the entire myotome.

We further injected embryos with adenoviruses encoding other cadherin mutants, AdV-cN390Δ and AdV-cN/CH(−) (Fig. 1 C). cN390Δ is known to be a dominant negative cadherin, since it interferes with the action of endogenous cadherins by competing for the interaction with the cytoskeletal proteins, resulting in the blocking of cadherin-mediated adhesion (Kintner, 1992; Fujimori and Takeichi, 1993; Levine et al., 1994). In AdV-cN390Δ–injected embryos, the DV expansion of the myotome was not inhibited,

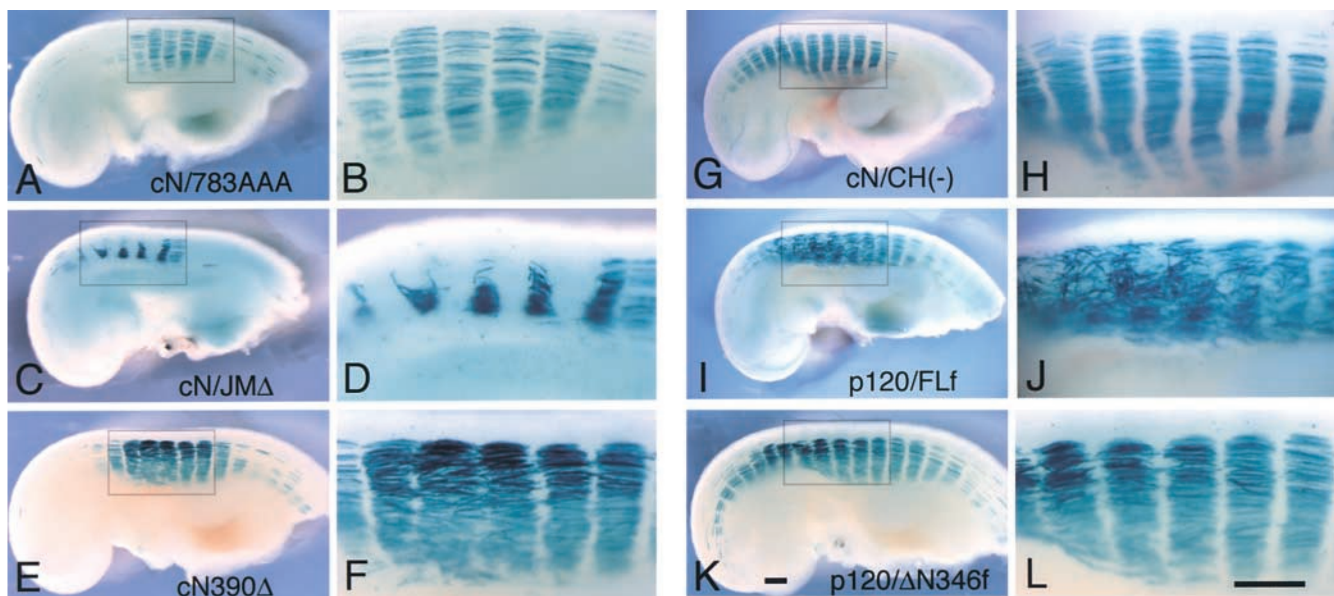

Figure 5. **Effects of expression of other cadherin or p120^{ctn} constructs on myotome development.** Embryos were coinjected with AdV-lacZ and AdV-cN/784AAA (A and B), AdV-cN/JMΔ (C and D), AdV-cN390Δ (E and F), AdV-cN/CH(−) (G and H), AdV-p120/FLf (I and J), or AdV-p120/ΔN346f (K and L). These were subjected to whole-mount X-gal staining at 72 h after injection. The triple alanine-substituted cadherin cN/784AAA had no effects on myotome morphogenesis (A and B). cN/JMΔ carrying a small deletion covering the 120^{ctn} binding site caused the same effects as cN/JM(−) (C and D). The dominant negative cadherin cN390Δ (E and F) and the CH domain–deleted cadherin cN/CH(−) (G and H) did not affect the DV expansion of myotome, although the former perturbed segmental boundaries. The NH_2 terminus–deleted p120^{ctn} had no effect on myotome formation (K and L), whereas the full-length p120^{ctn} affected the orientation of myofibers (I and J). Bars, 500 μm.

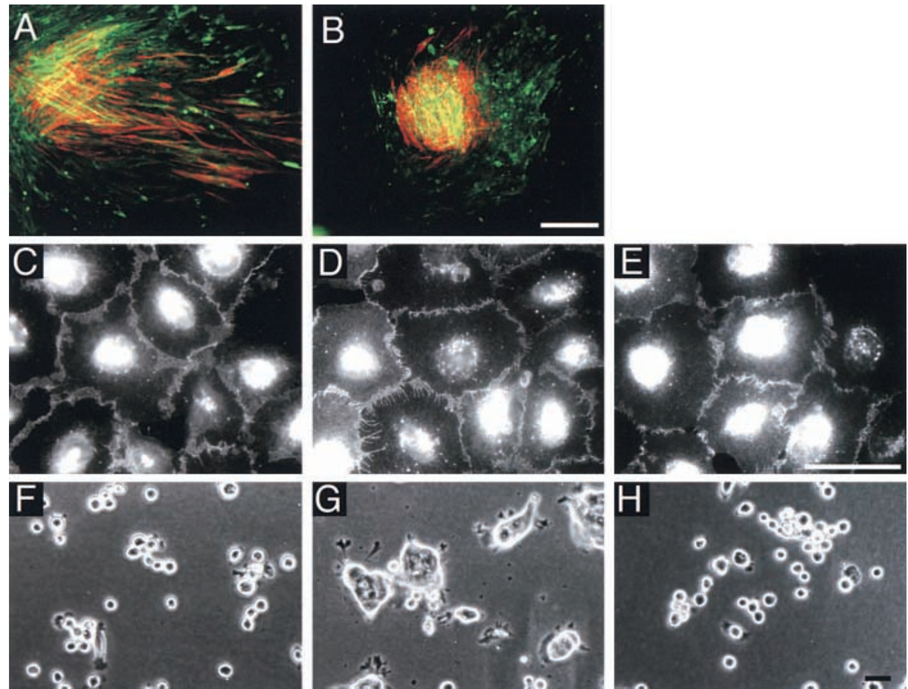

Figure 6. **Differential responses of cultured cells to mutant cadherins.** (A and B) Somites were isolated from embryos injected with AdV-Ncad (A) or AdV-cN/JMΔ (B) at 18 h after the injection and explanted onto a culture dish. After 2 d, the explants were double stained for the cadherin-attached tag (green) and MHC (red). MHC-positive myofibers expressing the control N-cadherin extensively spread, exhibiting a characteristic elongated morphology. In contrast, those expressing AdV-cN/JMΔ tended to stay as a cell clump. (C–E) Cadherin-deficient A431D cells were infected with AdV-Ncad (C), AdV-cN/JMΔ (D), or AdV-cN/784AAA (E), cultured for 2 d, and stained for the tagged cadherins. Although the control N-cadherin shows a lamellipodia-like pattern at cell–cell boundaries (C), cN/JMΔ displays filopodia-like spiny processes (D). cN/784AAA shows an intermediate distribution (E). (F–H) Effect of these cadherin expressions on Colo205 cells. JMΔ cadherin induced compact aggregation of Colo205 cells (G), but the others did not (F and H). Bars, 30 μm.

and also the rostrocaudal extension of myofibers took place almost normally. However, the boundaries between the somites became unclear, since myofibers in the individual somites often invaded into neighboring ones (Fig. 5, E and F). These results indicate that the inhibition of cadherin activity has severe disorganizing effects on myotome formation but in a way different from those of the JM(−) cadherin or $p120^{ctn}$ expression. On the other hand, AdV-cN/CH(−) expression had no apparent effect on myotome or myofiber patterning (Fig. 5, G and H).

In vitro behavior of myoblasts and other cells expressing N-cadherin mutants

To observe at the cellular level the behavior of myotome cells expressing cadherin mutants, we isolated somites from embryos that had received an AdV-Ncad or AdV-cN/JMΔ injection and explanted them individually onto culture dishes. They attached to and spread on the dishes. After 2 d in culture, these explants were subjected to double staining for the cadherin-attached tag and MHC. Every explant contained a cluster of MHC-positive myotome cells. In the control N-cadherin–expressing explants, the cluster of MHC-positive cells extensively spread, exhibiting a characteristic elongated morphology (Fig. 6 A). In contrast, MHC-positive cells expressing JMΔ cadherin tended to stay as three-dimensional clumps (Fig. 6 B). These phenotypes are consistent with those observed in the above in vivo experiments.

To obtain further insights into the action of the JM domain, we transfected A431D cells, a cadherin-deficient epithelial line (Lewis et al., 1997), with AdV-Ncad, AdV-cN/JMΔ, or AdV-cN/784AAA. The overall cell–cell association patterns were not particularly different among these transfectants. However, the patterns of the distribution of these molecules at cell–cell contacts were not identical among them when the cells were observed in confluent cultures. Cells expressing normal N-cadherin contacted each other with lamellipodia-like margins, and N-cadherin accumulated at their overlapping portions (Fig. 6 C). When cN/JMΔ was expressed, the cell–cell contacts expressing this molecule tended to develop spiny processes (Fig. 6 D). Cells transfected with cN/784AAA, encoding a triple alanine-substituted mutant unable to bind to $p120^{ctn}$, exhibited an intermediate contact morphology between the above two cases (Fig. 6 E). These observations support the idea that the JM domain, including the associated $p120^{ctn}$, play some regulatory role in cell junction formation consistent with previous observations (Thoreson et al., 2000).

We also transfected Colo205 cells with these three constructs. As reported previously (Aono et al., 1999), the expression of cN/JMΔ induced their compact aggregation, whereas that of control N-cadherin slightly affected the adhesion of these cells (Fig. 6, F and G). Interestingly, cN/784AAA expression also had no effect on the aggregation of Colo205 cells, indicating that the $p120^{ctn}$ binding to cadherin is not involved in the JM domain–dependent inhibition of the cadherin system in this cell line. Thus, the responses of cells to the deletion of the entire JM domain or to the specific blocking of the $p120^{ctn}$ binding to cadherin differ among cell types. For example, whereas myotome cells were similar to Colo205 cells in that both of them responded to cN/JMΔ but not to cN/784AAA, they were different in that myotome cell arrangement was not affected by the expression of $\Delta Np120^{ctn}$, a NH_2 terminus–deleted $p120^{ctn}$, but Colo205 aggregation was promoted by this construct (Aono et al., 1999). There seems to be a complex signaling system, containing active constituents which are different among cell types, to regulate the function of the JM domain.

Discussion

The JM domain of the cadherin cytoplasmic tail is highly conserved among species from vertebrates to invertebrates, implying an important function for it. Some biological activities of the JM domain were first suggested by experiments to test the effect of its overexpression in *Xenopus* embryos (Kintner, 1992). Later, the JM domain was found to be the site to which p120^{ctn} binds (Daniel and Reynolds, 1995; Shibamoto et al., 1995; Staddon et al., 1995). In the present study, we showed that myotome cells expressing JM domain–deficient cadherins could not undergo normal translocation along the DV axis, indicating that this domain is required for some cell rearrangement processes.

In normal development, myotome cells arise at the dorsomedial and ventrolateral lips of the expanding dermomyotome. The myotome cells derived from the dorsomedial lip turn around so as to migrate underneath the inner surface of the dermomyotome, and they subsequently differentiate into myofibers, elongating in a rostrocaudal direction and forming the epaxial muscles (Ordahl et al., 2001). When JM(−) cadherin was expressed, this morphogenetic cell rearrangement was inhibited, and myotome cells began to clump from the early stage of their differentiation. Late differentiating myotome cells joined this clump at more lateral positions. Our in vitro experiments to explant somites reproduced the in vivo phenomenon in which myotome cells remained clumped in somite cell colonies expressing JM(−) cadherin. This finding suggests that the clumping of myotome cells was not a consequence of some environmental changes, which could have been induced by JM(−) cadherin expression but brought about by their own changes in adhesive or other properties.

The results of these in vitro experiments were also important in excluding the possibility that JM(−) cadherin expression could have influenced Wnt signals coming from the neural tube. Wnts expressed in the roof plate of the neural tube are required for the development of the dorsomedial portion of the myotome (for review see Tajbakhsh and Cossu, 1997), and the overexpression of cadherins might have depleted β-catenin, a component necessary for Wnt signaling, resulting in a suppression of differentiation of the dorsomedial myotome. However, the neural tube was removed in the above experiments; nevertheless, the clumping behavior of myotome cells was observed. It should also be stressed that overexpression of other cadherin constructs having the β-catenin binding domain, such as the full-length N-cadherin, had no effects on myotome development, supporting the notion that the action of JM(−) cadherin cannot be interpreted in terms of β-catenin depletion.

We can discuss two possible mechanisms as to how the loss of the JM domain inhibited myotome expansion. The first possibility is that cells become stably and irreversibly connected together via the JM(−) cadherin and were unable to disrupt their contacts for proceeding to the translocation step. Concerning this idea, we should refer to our previous and present observation that when a JM domain–less cadherin, but not the full-length one, was ectopically expressed in Colo205 cells whose endogenous cadherin activity is physiologically suppressed, these cells acquired normal cadherin-dependent adhesiveness. This finding suggested that the JM domain exerts an inhibitory action on cadherin under particular physiological conditions and that in Colo205 cells it is constitutively active. In normal cells, the action of the JM domain should be controlled by certain signaling systems, and myotome cells might utilize such systems to loosen their mutual adhesion before translocation. Without this domain, myotome cells may not be able to control the cadherin adhesion machinery, resulting in stable clumping.

The second model assumes that the JM domain may have an activity to control cell motility. A previous study showed that the motility of WC5 cells was not affected by expression of an E-cadherin lacking the JM domain but was suppressed by expression of a mutant E-cadherin that retained the JM domain but lacked the carboxy half domain (Chen et al., 1997), suggesting that the JM domain has an activity to affect cell motility. Another study showed that a dominant negative N-cadherin impaired axon and dendrite growth and that this activity was elicited by the JM domain (Riehl et al., 1996). This again implies a role of the JM domain in motility and growth cone motility. Although these and our present observations are apparently not consistent with each other with respect to whether the JM domain inhibits or promotes cell motility, all of the findings support the idea that the JM domain has a role to regulate the nature of cellular motility. Different cell types may have distinct intracellular signaling environments in using the JM domain, exhibiting apparently opposite responses to this single signaling molecule as observed for other signaling systems such as growth cone guidance receptors (for review see Song and Poo, 2001). In fact, the above mentioned JM domain–dependent inhibition of axon growth was observed only for a limited group of neurons (Riehl et al., 1996). Thus, a role of the JM domain might be to control cell motility, and myotome cells with the overexpressed JM(−) cadherin might not have been able to move. In a similar context, we should also consider the hypothesis that the JM domain regulates β1-integrin function via the nonreceptor tyrosine kinase Fer, proposed previously (Lilien et al., 1999; Arregui et al., 2000). The JM domain could function for the regulation of integrin activity required for the migration of myotome cells.

Studies using cell lines provide some insights into the role of the JM domain. In the present study, we showed that A431D cells expressing the JM domain–deleted cadherin produced spiny processes containing this protein at their contacts. This observation supports the idea that the JM domain has a role in the regulation of cytoskeletal organization associated with cell adhesion. The next obvious question is what molecules represent the JM domain activity. p120^{ctn} is one of the molecules that have been identified as proteins that bind to the JM domain. In Colo205 cells, p120^{ctn} acts as a regulator of cadherin activity (Aono et al., 1999). In epithelial cells, this molecule seems to be required for normal cadherin-mediated cell–cell adhesion (Thoreson et al., 2000). Moreover, p120^{ctn} overexpression affects activities of small GTPases, inhibiting Rho but activating Rac and Cdc42 (Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). These findings suggest that p120^{ctn} regulates actin-based cytoskeletal organization and can be an actual player in the JM domain–associated biological phenomena.

However, in the present study overexpression of p120[ctn] and of its NH_2 terminus–deleted mutant had no effect on the DV expansion of myotome, and also a triple alanine substitution mutant of N-cadherin, which is unable to bind p120[ctn], had no effect on this phenomenon. These data suggest that this catenin is not involved in myotome expansion, at least as its cadherin-bound form. On the other hand, a deletion of 17 amino acid residues covering the p120[ctn] binding region in the JM domain could inhibit myotome expansion. These observations suggest the presence of other unidentified mechanisms or molecules that interact with this domain and may play a role in the above hypothetical mechanisms. The presence of such mechanisms are also supported by the other findings. First, the expression of cN/JMΔ cadherin in A431D cells resulted in a more profound phenotype than that seen with cN/784AAA; that is, the absence of p120[ctn] is not sufficient to explain the overall effect of cN/JMΔ cadherin expression. Second, in Colo205 cells cN/784AAA molecules could not induce their compact aggregation: this indicates that molecules other than p120[ctn] are responsible for inhibiting the cadherin system in this cell line. Such molecules could be shared by Colo205 and myotome cells for cadherin regulation. The role of ΔNp120[ctn] in inducing Colo205 compaction shown in our previous study (Aono et al., 1999) is possibly one of inhibiting the cadherin inhibitory action of the above hypothetical molecules. It was shown that presenilin-1 bound to the JM domain of E-cadherin, competing with p120[ctn] (Baki et al., 2001). Such molecules must be considered as a candidate for the regulator of JM domain functions, although it remains to be tested whether presenilin-1 is involved in myotome cell rearrangement.

Concerning p120[ctn] itself, we observed an interesting activity. Its overexpression had a dramatic effect on rostrocaudal elongation of myofibers, many of which were misdirected during the elongation when p120[ctn] was overexpressed. This phenomenon may be related to the anomalous cell shape changes observed when this molecule is overexpressed in cell lines in vitro (Reynolds et al., 1996; Anastasiadis et al., 2000; Noren et al., 2000; Grosheva et al., 2001). p120[ctn]-dependent regulation of small GTPases might be necessary for proper arrangement of myofibers.

The failure of the dominant negative cadherin to inhibit the DM expansion of myotome cells is consistent with the observation that a similar construct did not inhibit laminar cell migration in the neural retina (Riehl et al., 1996). These cells may use other classes of adhesion molecules for translocation, if they need such molecules. It should be noted that the border cells in the *Drosophila* ovary require DE-cadherin when they migrate within the nurse cell cluster (Oda et al., 1997; Niewiadomska et al., 1999). Cadherin dependency of cell migration or translocation may differ among cell types. On the other hand, despite the inability of the dominant negative cadherin to block the DM expansion of myotome this construct perturbed the formation of boundaries between somites. This phenomenon can be ascribed to the disruption of epithelial somitic structure by the dominant negative cadherin (unpublished data). Probably, in normal development individual somites maintain their structural independence by forming closed epithelial units, and loss of such structures would result in the mixing of cells between neighboring somites. This finding indicates how important the epithelial segmentation is for somites to organize the future metameric body structure.

In summary, we showed that cell rearrangement involved in myotome development requires a regulation via the JM domain of the cadherin cytoplasmic tail. The role of this domain is perhaps to confer reversibility on cadherin-dependent adhesion or to regulate cell motility. The JM domain–associated protein p120[ctn] was apparently not involved in this process, but this protein seems to be important for later phases of myotome development. To further understand these complicated actions of the JM domain and its partner, we need further detailed analysis of the signaling system associated with these molecules. It is also intriguing to study whether the cadherin-mediated regulation of cell rearrangement found in the myotome can be generalized to other morphogenetic processes.

Materials and methods

Plasmid construction

For construction of cN/JMΔ, carrying a 17 amino acid deletion (YDEEGGGEEDQDYDLSQ) in the JM domain of the chicken N-cadherin, cDNA fragments encoding 1–778 and 797–912 amino acids were amplified by PCR using the primer sets 5′-GTTAACACCATGTGCCGGATAGCGGGAAC-3′ and 5′-AGATCTTTTCAGAATGTTGTCCCTCAC-3′, and 5′-AGATCTCAGCAGCCTGACACTGTA-3′ and 5′-GTCGACGTCATCACCTCCACCGTACA-3′, respectively. Obtained fragments were ligated through the underlined BglII sites. To construct cN/784AAA containing triple alanine substitutions for 784GGG, we conducted PCR-based site-directed mutagenesis. For generation of the mutated cDNA fragments corresponding to 1–784 and 785–912 amino acids of cN/784AAA, the following primer sets were used: 5′-GTTAACACCATGTGCCGGATAGCGGGAAC-3′ and 5′-ATCCTGATCTTCTTCAGCGGCCGCTTCTTCATCATATTT-3′, and 5′-AAATATGATGAAGAAGCGGCCGCTGAAGAAGATCAGGA-3′ and 5′-GTCGACGTCATCACCTCCACCGTACA-3′, respectively. Ligation of the obtained two fragments through the NotI sites (underlined) generated the cDNA of cN/784AAA. To construct the expression vectors for cN/JMΔ and cN/784AAA fused with the EGFP tag, each cDNA fragment was subcloned into pCA-EGFP-pA by use of a SalI linker. Generation of other constructs was reported previously (Nakagawa and Takeichi, 1998; Aono et al., 1999).

Construction of recombinant adenoviruses

Construction of recombinant adenoviruses expressing cN/JMΔ-EGFP and cN784AAA-EGFP was performed as reported previously (Moriyoshi et al., 1996; Nakagawa and Takeichi, 1998). Briefly, HEK293 cells in a 35-mm collagen-coated dish (Iwaki) were cotransfected with 0.5 μg of viral genome fragments and 1.0 μg of linearized shuttle vector plasmids by use of Effectene (QIAGEN). On the next day, the cells were replated in a 96-well plate (Iwaki) and cultured for 10–15 d. EGFP-positive plaques were collected under a fluorescence microscope and recloned if necessary. Finally, we obtained AdV-cN/JMΔ and AdV-cN/784AAA. Construction of other recombinant adenoviruses, AdV-Ncad, AdV-cN/JM(−), AdV-cN/CH(−), AdV-cN390Δ, AdV-p120/FLf, and AdV-p120/ΔN346f was reported previously (Nakagawa and Takeichi, 1998; Aono et al., 1999). After amplification and purification, 10^{10} pfu virus solutions were prepared and stored at −80°C until needed.

In ovo viral infection

Fertile chicken eggs were obtained from Yamagishi's Farm (Kyoto, Japan), and incubated at 38°C for 48 h to allow embryos to develop to stage 12 (16 pairs of somite). Virus solutions were diluted to 10^9 pfu with a sterile PBS to which Fast Green (Sigma-Aldrich) was added to give a final concentration of 0.2%. The tip of a pulled glass pipette was broken and filled with the diluted virus solution. Using a micromanipulator (Narishige), we transferred ~10 nl of virus solution into the segmental plate region with air pressure. For effective infection, it was important to spend ~2 min for each injection. Injected embryos were further incubated at 38°C until they were fixed. Upon examination of the injected embryos, we discarded those suspected of excessive viral infection, since this tended to affect the viability

of cells, causing nonspecific effects on embryonic morphogenesis. To determine optimal infection levels, we prepared a dilution series of control viral vectors encoding *lac-Z* and used them for injection. Higher viral titers, which cause developmental defects, were thus checked and not used in experiments. The relative titers of the viral vectors were also compared by Western blots of the recombinant proteins expressed in A431D or A431 cells (Fig. 1 E).

Immunohistochemistry

For whole-mount X-gal or immunostaining, embryos were dissected in PBS and fixed with 4% PFA in PBS for 2 h at 4°C. For cryosections, embryos were fixed with ice-cold Bouin's solution (Sigma-Aldrich) for 10–60 min. After having been washed extensively with PBS, embryos were immersed in a series of sucrose solutions (12, 15, 18, and 30% sucrose in PBS), embedded in Tissue Tek (Miles), and frozen with dry ice. Cryosections of 18-μm thickness were collected on PLL-coated glass slides. The procedure of immunostaining was described previously (Nakagawa and Takeichi, 1998). For immunostaining of cultured cells or somite explants, the cells or explants were washed three times with PBS, fixed with 4% PFA in PBS for 5 min at room temperature, and then permeabilized with Triton X-100 (0.25% in TBS containing 1 mM $CaCl_2$) for 5 min at 37°C. Fluorescence signals were monitored by use of a ZEISS Axiophoto microscope fitted with a CCD camera (Photometrics) connected to a computer for digital image capture. Whole-mount samples were viewed by use of a Leica MZFLIII dissecting microscope, and images were captured by a color CCD camera (Axiocam; ZEISS). For processing of digital images, Adobe Photoshop® 5.5 was used.

Antibodies

The following antibodies were used: mouse anti-$p120^{ctn}$ monoclonal antibody (Transduction Laboratories), rabbit anti–chicken N-cadherin polyclonal antibodies (Hatta et al., 1988), rabbit anti-FLAG polyclonal antibodies (Santa Cruz Biotechnology), mouse anti-GFP monoclonal antibody clones 7.1 and 13.1 (Roche Molecular Biochemicals), rabbit anti-GFP polyclonal antibodies (CHEMICON), mouse anti-MHC monoclonal antibody MF20 (Developmental Studies Hybridoma Bank), rabbit anti–β-catenin polyclonal antibodies (a gift from Dr. Sayumi Shibamoto, Setsunan University, Osaka, Japan), goat Alexa Fluor 488/594–conjugated anti–mouse or –rabbit IgG (Molecular Probes, Inc.), and sheep HRP-conjugated anti–mouse, –rat, or –rabbit IgG (Amersham Pharmacia Biotech).

Somite explant culture

18 h after viral infection, embryos were collected, dissected in PBS, and then placed in a pancreatin solution (GIBCO BRL) for 10 min at room temperature. Tissues surrounding somites were removed with a chemically sharpened tungsten wire, and isolated somites V–VIII were transferred into the culture medium. For explant cultures, S-clone SF-03 medium (Sanko) conditioned by L-cells for 2 d and supplemented with 10% FCS was used. Individual somites were placed separately on a 35-mm collagen-coated culture dish, each in a 5-μl drop of the culture medium, and incubated for 1 h at 37°C in a CO_2 incubator to allow them to attach to the substrate. Then, 1.5 ml of the same culture medium was added, and the explants were further incubated for 48 h and finally processed for immunostaining.

Cell culture and viral infection

A431D (Lewis et al., 1997), and its original line A431, and Colo205 (Semple et al., 1978) cells were cultured with DH10 (a 1:1 mixture of DME and Ham's F12 supplemented with 10% FCS). For viral infection, A431D or A431, and Colo205 cells were treated with 5 and 50 moi viral solution for 2 h, respectively. After having been washed with DH10, the cells were cultured for 48 h and then subjected to immunoblotting or immunostaining.

Immunoprecipitation

For immunoprecipitation, cells were lysed with the lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM $CaCl_2$, and 0.5% NP-40) containing protease inhibitor cocktails (Roche Molecular Biochemicals). Cell lysates were cleared by centrifugation, incubated with anti–chicken N-cadherin rabbit antibodies (Hatta et al., 1988) and then with protein G–Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4°C. Beads were washed with the lysis buffer, and associated proteins were dissolved in the SDS-PAGE sample buffer (125 mM Tris-HCl, pH 7.6, 2% SDS, 20% glycerol, 0.002% BPB, and 5% 2-mercaptoethanol). For Western blotting, these samples were electrophoresed through a 7.5% SDS-PAGE gel. Proteins were blotted onto a nitrocellulose membrane and detected by the ECL system (Amersham Pharmacia Biotech) as described previously (Watabe-Uchida et al., 1998).

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