Wnt signaling from the dorsal neural tube is required for the formation of the medial dermomyotome

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SUMMARY

Signals originating from tissues surrounding somites are involved in mediolateral and dorsoventral patterning of somites and in the differentiation of the myotome. Wnt-1 and Wnt-3a, which encode members of the Wnt family of cystein-rich secreted signaling molecules, are coexpressed at the dorsal midline of the developing neural tube, an area adjacent to the dorsomedial portion of the somite. Several lines of evidence indicate that Wnt-1 and Wnt-3a have the ability to induce the development of the medial and dorsal portion of somites, as well as to induce myogenesis. To address whether these Wnt signalings are really essential for the development of somites during normal embryogenesis, we investigated the development of somites in mouse embryos lacking both Wnt-1 and Wnt-3a. Here we demonstrate that the medial compartment of the dermomyotome is not formed and the expression of a lateral dermomyotome marker gene, Sim-1, is expanded more medially in the absence of these Wnt signalings. In addition, the expression of a myogenic gene, Myf-5, is decreased at 9.5 days post coitum whereas the level of expression of a number of myogenic genes in the later stage appeared normal. These results indicate that Wnt-1 and Wnt-3a signalings actually regulate the formation of the medial compartment of the dermomyotome and the early part of myogenesis.

Key words: Wnt, Pattern formation, Somite, Dermomyotome, Medial lip, Myotome, Myogenesis, Mouse

INTRODUCTION

During vertebrate embryogenesis, the paraxial mesoderm gives rise to segmented epithelial spheres called somites. The ventral part of these somites subsequently becomes mesenchymal to form the sclerotome, which constructs the axial skeleton. In contrast, the remaining dorsal epithelial part becomes the dermomyotome, which gives rise to dermis and muscle (Christ and Ordahl, 1995). The dermomyotome is patterned along its mediolateral axis into medial, central and lateral compartments. Cells that leave the medial edge of the dermomyotome (the medial lip) spread beneath it and form the myotome that gives rise to axial skeletal muscle (epaxial muscle). Cells in the lateral compartment of the dermomyotome migrate to give rise to the body wall and limb musculature (hypaxial muscle; Selleck and Stern, 1991; Ordahl and Le Douarin, 1992). Most cells in the central compartment form the dermis.

Signals from adjacent tissues play important roles in the initial patterning of somites along the dorsoventral and mediolateral axes. Commitment of cells in somites occurs after somite formation in response to external cues (Ordahl and Le Douarin, 1992; Aoyama and Asamoto, 1988; Christ et al., 1992; Dietrich et al., 1997). Several lines of evidence have indicated that Sonic Hedgehog, secreted from the notochord and floor plate, acts as a ventralizing signal (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Chiang et al., 1996) and also can act as a medializing signal (Kos et al., 1998). In contrast, signals from the surface ectoderm and the dorsal neural tube can induce formation and/or maintenance of the dermomyotome (Brand-Saberi et al., 1993; Pourquie et al., 1993; Kuratani et al., 1994; Fan and Tessier-Lavigne, 1994; Spence et al., 1996). Signals from the dorsal neural tube are also involved in medialization of the dermomyotome; whereas BMP-4, which is secreted from the lateral plate mesoderm, can lateralize it in the chick embryo (Marcelle et al., 1997; Hirsinger et al., 1997; Spence et al., 1996).

Wnt-1 and Wnt-3a are likely candidates as signals from the dorsal portion of the neural tube to direct dorsalization and medialization of somites (Marcelle et al., 1997; Hirsinger et al., 1997). During the period of initial somite development in the mouse, these Wnt genes are expressed in the dorsalmost portion of the neural tube (roof plate) from 8.5 days post coitum (d.p.c.) (Wilkinson et al., 1987; McMahon et al., 1992; Parr et al., 1993). Recent evidence indicated that Wnt-1 and Wnt-3a have the ability to direct dorsalization of somites and medialization of the dermomyotome. Wnt-1 and Wnt-3a are also known to be able to maintain and induce dermomyotome marker expression in presomitic mesoderm (Fan et al., 1997; Capdevila et al., 1998). Wnt-1-expressing cells can induce expression of medial dermomyotome marker genes, Wnt-11.
and noggin, and can rescue medial somite patterning in embryos in which the neural tube was ablated (Marcelle et al., 1997; Hirsgen et al., 1997; Reshef et al., 1998).

Since Wnt-1 and Wnt-3a have similar activity in vitro (Wong et al., 1994), no obvious defect in somite patterning was observed in either Wnt-1 or Wnt-3a single mutant embryos, probably due to functional redundancy between these two genes (McMahon and Bradley, 1990; Thomas and Capecchi, 1990, Takada et al., 1994). In fact, in embryos lacking both Wnt-1 and Wnt-3a, we have observed a severe loss of cells in the neural crest and the dorsal hindbrain, which was not seen in either single mutant (Ikeya et al., 1997). To address whether these Wnt signalings are really essential for somite patterning, in the present study, we have investigated the development of somites in Wnt-1/Wnt-3a compound mutant embryos. Here we show that both Wnt signals play essential roles in the formation of the medial compartment of the dermomyotome and the differentiation of myotomal cells.

MATERIALS AND METHODS

Generation and collection of Wnt-1−/−; Wnt-3a−/− double homozygous mutant embryos

Generation of mice heterozygous for null alleles of Wnt-1 and Wnt-3a was described previously (McMahon and Bradley, 1990; Takada et al., 1994). Compound heterozygotes (on a predominantly 129/Sv background) were intercrossed to recover compound homozygous mutants. Genotypes were initially confirmed by genomic Southern hybridization and PCR.

Histology

Embryos were dissected in phosphate-buffered saline (PBS), fixed in Bouin’s fixative for 2 hours, and processed for sectioning and hematoxylin-eosin staining as previously described (Takada et al., 1994).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out according to the technique described previously (Wilkinson, 1992; Shimamura et al., 1995; Yoshikawa et al., 1997) with some modifications. Briefly, embryos were collected in ice-cold PBS, fixed for overnight at 4°C in 4% paraformaldehyde/PBS and stored in methanol at −20°C until used. Prior to hybridization, the embryos were bleached in 5:1 methanol/30% hydrogen peroxide for 5 hours at room temperature, then rehydrated by passage through graded series of methanol/PBS. Thereafter they were treated with 20 μg/ml (9.5 d.p.c. embryos) or 60 μg/ml (10.5 d.p.c. embryos) of protease K/PBS for 10 minutes at room temperature. The following steps of procedure were the same as described earlier (Yoshikawa et al., 1997).

The following probes were used for the whole-mount in situ hybridization studies: En-1 (Davis et al., 1991), Myf-5 (Ott et al., 1991), MyoD, myogenin (Sassoon et al., 1989), noggin (McMahon et al., 1998), Notch 2 (Williams et al., 1995), Pax-1 (Deutsch et al., 1988), Pax-3 (Goulding et al., 1991), Sim-1 (Fan et al., 1996), Wnt-6 (Parr et al., 1993) and Wnt-11 (Christiansen et al., 1995; Kispert et al., 1996).

Cell proliferation and cell death

Embryos were collected at 9.5 d.p.c. after intraperitoneal injection of pregnant females with 50 μg/g body weight of 5-bromo-2′-deoxyuridine (BrdU) and the mice were killed 2 hours later. The embryos were fixed for 2 hours in 4% paraformaldehyde in PBS at 4°C and subsequently embedded in Tissue Tek OCT Compound (Miles Scientific) after passage through a graduated series of sucrose solutions. Serial cryosections were made at a thickness of 6 μm, rehydrated and then either assayed for BrdU incorporation (Miller and Nowakowski, 1988) or for apoptotic cell death by the TUNEL procedure (Gavrieli et al., 1992).

RESULTS

The medial compartment of the dermomyotome is missing in Wnt-1/Wnt-3a compound homozygous embryos

To investigate whether development of somites is defective in Wnt-1/Wnt-3a compound homozygous embryos, we first observed the morphology of the somites in histological sections. Since lack of the Wnt-3a gene causes posterior truncation at the forelimb level (Takada et al., 1994), we observed transverse sections at the axial level of the cervical region of embryos from 8.5 d.p.c. to 11.0 d.p.c. By 8.5 d.p.c., epithelial spheres of somites have already formed normally at this axial level in Wnt-1/Wnt-3a compound homozygous embryos (Fig. 1A,B). At 10.0 d.p.c., the layer of columnar epithelial cells of the dermomyotome of the wild-type embryo had already formed beneath the surface ectoderm and cells...
from the medial lip of the dermomyotome have already started to involute under the dermomyotome to form the myotome; whereas the sclerotomal cells still remained ventral to the dermomyotome. While the formation of the dermomyotome appeared normal in Wnt-1 homozygous mutants and Wnt-3a homozygous mutant embryos, as in the wild-type embryos (Fig. 1C), the medial lip of the dermomyotome was missing and myotome formation was poorly organized in Wnt-1/Wnt-3a compound homozygous embryos (Fig. 1D). At 11.0 d.p.c. the myotome, which was observed as a condensed cell layer in the wild type and in either single mutant (Fig. 1E-G), did not form normally in the Wnt-1/Wnt-3a compound homozygous embryo; i.e. only small clusters of myotomal cells appeared in the compound mutant (Fig. 1H).

To identify the molecular characteristics of the reduced dermomyotome in the compound mutant embryos, we performed in situ hybridization to study the expression of a variety of molecular markers. Since it was reported that the expression of Pax-3, which is normally expressed in the dermomyotome, can be induced by the neural tube and Wnt-1/Wnt-3a signalings (Fan and Tessier-Lavigne, 1994; Fan et al., 1997; Capdevila et al., 1998), it might be possible that cells in the dorsal somite did not have the molecular characteristics of the dermomyotome in the compound mutant although these cells appeared to form epithelial cell layers like those in the normal dermomyotome at 10.0 d.p.c. (Fig. 1B). To address this possibility, we examined the expression of Pax-3. In the compound mutant embryos at 9.5 d.p.c., Pax-3 was expressed in the dorsal somites, although the number of cells that expressed this gene was slightly decreased, suggesting that the dorsal somites have the molecular characteristics of the dermomyotome (Fig. 2B,C). In contrast, the expression of a sclerotome marker Pax-1 was not obviously changed in the compound mutants, indicating that the dorsoventral patterning of somites as a whole did not appear to be changed (Fig. 2D,E).

To determine whether the absence of the medial dermomyotome, observed in histological sections, was caused by the lack of cells forming the medial lip of the dermomyotome, we next examined the expression of a number of molecular markers that are normally expressed in this region. The expression of Wnt-6, which is normally observed in the medial lip of the dermomyotome at 9.5 d.p.c. (Fig. 3B,D) (Parr et al., 1993), was completely missing in the compound mutant embryo (Fig. 3C,E). Similarly, expression of other genes normally expressed in the medial lip of the dermomyotome, i.e. Notch 2, noggin and Wnt-11, were not detected in the compound mutant embryo at 9.5 d.p.c. (Fig. 3F-K) (Williams et al., 1995; Christiansen et al., 1995; Kispert et al., 1996; McMahon et al., 1998). These results indicate that the cells that normally form the medial lip were absent in the Wnt-1/Wnt-3a compound mutant embryos.

The lack of the medial lip could result from defective cell proliferation and/or cell survival in compound mutant embryos. Cell proliferation and cell death in the compound mutant embryos at 9.5 d.p.c., when we can observe the formation of the dermomyotome, was examined using bromodeoxyuridine incorporation and TUNEL staining, respectively. However, no obvious difference was observed in either cell proliferation or cell death between wild-type and compound mutant embryos (data not shown).

Mediolateral patterning of the dermomyotome is defective in Wnt-1/Wnt-3a compound homozygous embryos

Lack of the medial compartment of the dermomyotome may affect mediolateral patterning of the dermomyotome. To examine this possibility in Wnt-1/Wnt-3a compound homozygous embryos, we performed in situ hybridization to analyze the expression of several genes whose expression is characteristic along the mediolateral axis in the dermomyotome.

En-1 is normally expressed in the central compartment of the dermomyotome at 9.5 d.p.c. (Fig. 3L). However, the expression of En-1 was missing in the compound mutant embryos (Fig. 3M). On the contrary, Sim-1, which encodes a bHLH transcription factor, is normally expressed in the lateral half of the dermomyotome at 10.5 d.p.c. (Fig. 4B,D) (Pourquie et al., 1996; Ema et al., 1996; Fan et al., 1996). In the
compound mutant embryos, Sim-1 expression was expanded more medially and, as a consequence, most of cells in the dermomyotome expressed it (Fig. 4C,E). These results indicated that the dermomyotome in the compound mutant embryos was lateralized in addition to lacking the medial lip.

**Defects in the expression of a myogenic gene in Wnt-1/Wnt-3a compound homozygous embryos**

Histological analysis of the compound mutant embryos indicated that the formation of the myotome was impaired in addition to the lack of the medial dermomyotome. To investigate the development of the myotome in the compound mutant embryo, we examined the expression of myogenic genes by in situ hybridization.

A number of myogenic bHLH genes are expressed coordinately in the process of myogenesis in the mouse embryo (Cossu et al., 1996). Among these genes, Myf-5 is first expressed in the medial part of the dermomyotome from 9.5 d.p.c. in the normal mouse development. In contrast, MyoD, another myogenic gene, starts to be expressed one day later in the myotome. Expression of Myf-5 was reduced at 9.5 d.p.c. in the compound mutant embryos, whereas it was normal in the embryos of the other genotypes (Fig. 5A,B). However, its expression recovered to the normal level at 11.0 d.p.c., although the number of cells that expressed Myf-5 was reduced, probably due to the decrease in the number of cells in the myotome (Fig. 5C,D). Similarly, the level of expression of MyoD at 11.0 d.p.c. in the compound mutant was almost normal (Fig. 5E-H). The level of expression of another myogenic gene, myogenin, appeared also to be normal at 11.0 d.p.c. in the compound mutant embryos (data not shown). Thus, Wnt-1/Wnt-3a signalings appear to be essential for the expression of Myf-5 in early stages, whereas their action is not necessary for the differentiation of myotomal cells in later stages.

**DISCUSSION**

**Wnt signaling from the dorsal midline of the neural tube regulates the formation of the medial dermomyotome**

There have been a number of studies indicating that signals secreted from the dorsal neural tube are involved in the patterning of somites along their dorsoventral and mediolateral axes (Spence et al., 1996; Marcelle et al., 1997; Hirsinger et al., 1997; Pourquie et al., 1996). However, the role of signaling molecules secreted from the dorsal neural tube in the normal development of somite is unclear because of the lack of a loss-of-function analysis of these molecules. In this study, we demonstrated that Wnt-1/Wnt-3a signalings are necessary for the formation of the medial dermomyotome by analyzing Wnt-1/Wnt-3a compound mutants. Histological analysis and expression of marker genes such as Wnt-6, Wnt-11, Notch-2 and noggin revealed that the medial compartment of the dermomyotome was clearly missing in Wnt-1/Wnt-3a compound mutant embryos.

There are at least three possible mechanisms to explain how Wnt-1/Wnt-3a signalings regulate the formation of the medial dermomyotome. One possible mechanism is that Wnt-1/Wnt-3a signaling molecules, secreted from the dorsal neural tube, regulate the proliferation of adjacent cells, including the dorsomedial compartment of the somite. Several lines of evidence indicate that Wnt-1/Wnt-3a signalings support cell proliferation. Wnt-1 expression causes excess cell proliferation at confluence, in addition to morphological transformation, in mammary epithelial cells in vitro (Brown et al., 1986;
Rijsewijk et al., 1987; Bradley and Brown, 1995). A transgenic experiment in which Wnt-1 was expressed ectopically within the CNS demonstrated that this signaling molecule can act as a potent mitogen during gestation (Dickinson et al., 1994). Recently, we also found that the number of dorsolateral progenitor cells of the hindbrain was remarkably reduced in the Wnt-1/Wnt-3a compound mutant embryos, suggesting that Wnt signaling molecules secreted from the dorsal neural tube regulate the expansion of adjacent cells in the neural tube (Ikeya et al., 1997). However, in the present study, bromodeoxyuridine incorporation did not reveal any obvious defect in cell proliferation in the dermomyotome of Wnt-1/Wnt-3a compound mutant embryos at 9.5 d.p.c. Thus, even if Wnt-1/Wnt-3a signalings may promote cell proliferation at the medial lip, their effects would not be so obvious that we could detect their dysfunction by bromodeoxyuridine incorporation.

Another possible mechanism is that Wnt-1/Wnt-3a signalings regulate cell survival in the medial lip. However, in this study, we did not observe any discernible change in cell death in the dermomyotome of the compound mutant embryo at 9.5 d.p.c. by TUNEL staining. We cannot exclude the possibility that TUNEL staining is not sensitive enough to detect any difference in cell death between in the wild-type and in the Wnt-1/Wnt-3a compound mutant. Extensive cell death analysis in the future should reveal whether this is the case.

A third possible mechanism is that Wnt-1/Wnt-3a signalings are involved in the patterning of the somite and that the absence of these signalings would lead to the transformation of the medial dermomyotome to another fate. For instance, if these Wnt molecules regulate the dorsoventral patterning of somites, the loss of these signals might cause the transformation of presumptive dermomyotomal cells to sclerotome cells in a part of the somite, located close to the dorsal neural tube. Indeed, the dorsal neural tube and also Wnt-expressing cells have been shown, by use of in vitro explant cultures of chick presomitic...
mesoderm and by ectopic expression in ovo, to have the ability to maintain and induce dermomyotome marker expression (Fan et al., 1997; Capdevila et al., 1998). In this study, we observed no discernible change in sclerotome formation, as assessed by the expression of *Pax-1* (Fig. 2D, E). This result appears to show that Wnt-1/Wnt-3a signalings have little or no critical role in the dorsoventral patterning of the somite as a whole. However, by this analysis, it is very difficult to examine whether or not a slight change in the number of cells occurred in a localized area of the sclerotome, for instance, an area close to the neural tube. Thus, to address this issue rigorously, extensive cell lineage analysis would be required.

The question arises as to whether Wnt-1/Wnt-3a signalings act directly on the paraxial mesoderm or indirectly by inducing another signal that may affect the development of the somite. Since several signaling molecules, for instance BMPs and Noggin, are expressed in the dorsal neural tube, it might be possible that a lack of Wnt signaling results in altered production of these molecules. However, expression of *Bmp-7* (Ikeya et al., 1997) and *noggin* (data not shown) was induced normally in the compound mutant embryo. Furthermore, examination of the distribution of a number of regionally expressed markers, including *Lmx1b, Math1, Pax-5, Dbx* and *Pax-6*, revealed that the dorsoventral polarity within the neural tube is also normal in the compound mutant (Ikeya et al., 1997). Thus it seems unlikely that defective dermomyotome development resulted from a secondary effect on other signals from the neural tube. Recent evidence that Wnt-1-producing cells can induce medial lip marker genes, i.e. Wnt-11 and noggin, in the somite also supports the idea that the Wnt signal from the dorsal neural tube directly specifies the dorsomedial somite (Marcelle et al., 1997; Hirsinger et al., 1997; Reshef et al., 1998).

In the compound mutant, we observed no expression of various genes that are normally expressed in the medial lip, i.e. *Wnt-6*, *Wnt-11*, *Notch-2* and *noggin* (Fig. 3). Since Wnt-1-producing cells can induce expression of at least some of them, e.g. *Wnt-11* and *noggin* (Marcelle et al., 1997; Reshef et al., 1998), and the temporal expression of these genes in the medial lip correspond well to the expression of *Wnt-1/Wnt-3a* in the neural tube (Parr et al., 1993; Kispe et al., 1996; Christiansen et al., 1995; Williams et al., 1995), it would be plausible that these genes in the medial lip are induced by Wnt-1/Wnt-3a signalings and their products may regulate the development of the somite during normal embryogenesis. It is also suggested that loss of function of these genes results in some of the defects observed in the compound mutants.

*Wnt-6* and *Wnt-11* are members of the Wnt gene family, which comprises at least 17 genes in the mouse. Based on assays carried out with mammalian cell lines and *Xenopus* embryos, the Wnt genes can be grouped into at least two distinct classes, *Wnt-1* and *Wnt-5a* classes (Du et al., 1995; Wong et al., 1994). Since Wnt-6 and Wnt-11 have activities similar to those of Wnt-5a and not to those of Wnt-1 and Wnt-3a (i.e. Wnt-6 has no transforming activity and Wnt-11 can alter morphogenetic movement in *Xenopus* eggs), these two Wnt signalings seem to be categorized into the Wnt-5a class. Thus, it seems plausible that Wnt-1/Wnt-3a signalings induce another class of Wnt genes, the Wnt-5a class, in the medial lip and that the induced Wnt-5a class genes, *Wnt-6* and *Wnt-11*, regulate the somite development. Interestingly, some other Wnt-5a class genes, i.e. *Wnt-4* and *Wnt-6*, are also expressed in the surface ectoderm, which is known to secrete signals to dorsalize the somite. Actually, tissue culture cells expressing these Wnt genes can maintain and induce dermomyotome marker expression in presomitic mesoderm explants (Fan et al., 1997). At present, it is not certain whether Wnt genes expressed in the medial lip and in the surface ectoderm play a similar role or not. In contrast, *Wnt-4* and *Wnt-3*, another member of the Wnt-1 class genes, are expressed at the dorsal neural tube, although it is not certain whether their expression may have a role in somite development. Intensive genetical studies of these Wnt genes should reveal their roles in somite development.

We demonstrated that the expression of *Sim-1*, which is normally expressed in the lateral compartment of the dermomyotome, was expanded medially in the compound mutant while that of *En-1*, which is normally expressed in the central compartment of the dermomyotome, was lost completely, in addition to the loss of the expression of medial markers (Figs 3, 4). These results indicate that Wnt signaling from the dorsal neural tube also regulates mediolateral patterning directly or indirectly. Recently, it was shown that ectopically expressed Noggin signaling could induce medial dermomyotome formation by antagonizing BMP signaling, which is secreted from the lateral plate and specifies the lateral dermomyotome (Hirsinger et al., 1997; Capdevila and Johnson, 1998). Since the lack of Wnt-1/Wnt-3a signaling led to the loss of the medial lip, where *noggin* is normally expressed, it is plausible that Wnt signaling regulates medial dermomyotome specification indirectly by antagonizing BMP signaling in the medial lip.

**Myogenesis as a regulative developmental process**

Recently, cell fate analysis has demonstrated that the medial compartment of the dermomyotome is one of the origins of myotomal cells in the chick embryo (Denetclaw et al., 1997). Thus, it has been expected that the lack of the medial lip of the dermomyotome would affect myogenesis. Indeed, in this study, we obtained several lines of evidence to indicate that the development of the myotome was defective in the *Wnt-1/Wnt-3a* compound mutant embryo, which lacks the medial lip. To understand the process of the myotome development that occurs in the medial lip, it would be important to characterize the molecular events that are abnormal in the compound mutant embryo.

We demonstrated that the initial expression of *Myf-5*, a myogenic bHLH gene, was decreased in the compound mutant at 9.5 d.p.c. whereas its expression was recovered to the normal level by 11.0 d.p.c. (Fig. 5A-D). In contrast, the level of the expression of another myogenic bHLH gene, *MyoD*, which is normally expressed first at 10.5 d.p.c., was normal (Fig. 5E-H). These results indicated that the two myogenic bHLH genes, *Myf-5* and *MyoD*, are differently regulated in the myogenesis of the mouse and that, whereas Wnt signaling is initially needed for sufficient expression of *Myf-5*, this insufficient expression can be rescued in a later stage probably by another myogenesis-inducing signal. Differential regulation of the expression of *Myf-5* and *MyoD* was also observed earlier in an in vitro experiment. In the chick, cells from the medial half of the segmental plate, when cultured in the presence of the neural tube, activated *Myf-5* expression, whereas cells from the lateral half, cultured with their own surface ectoderm, activated *MyoD*.
expression (Cossu et al., 1995). Subsequently, the great majority of myogenic cells came to express Myf-5 and MyoD in this experiment. Thus, a signal originating from the surface ectoderm is likely to activate MyoD expression and, subsequently or secondarily, Myf-5 expression, in the compound mutant embryos.

Taken together, it would be appropriate to suggest that, although Wnt-1/Wnt-3a signaling from the dorsal neural tube initially activates Myf-5 in the medial somite, some unknown signal from the surface ectoderm or from the dorsal neural tube in later stage is sufficient for the activation of MyoD and Myf-5 expression and the progression of myotome development. Thus, there is a functional redundancy in myogenesis between inducing signals. Such a redundant mechanism would guarantee that myogenesis would proceed normally during vertebrate development.

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