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Transient Expression of Mouse Pro-α3(V) Collagen Gene (*Col5a3*) in Wound Healing

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The α3(V) chain is poorly characterized among type V collagen chains. Pro-α3(V) collagen is expressed in newly synthesized bone as well as in the superficial fascia of developing muscle. Present study examined the expression in a mouse model of wound healing. Real-time reverse transcriptase polymerase chain reaction and in situ hybridization revealed transient expression of pro-α3(V) chain at a lower level than other fibrillar collagen genes after injury. Immunohistochemistry showed a similar expression pattern in the injured skin. In addition, electron microscopy showed that pro-α3(V) chain was localized in the amorphous nonfibrillar region, but not in fine or dense fibrils. The pro-α3(V) chain co-localized with heparan sulfate, which appeared in the skin after injury and might bind via an acidic segment of the pro-α3(V) chain. The matrix containing the pro-α3(V) chain may therefore be needed for the initiation of wound healing.

Keywords: type V collagen, wound healing, gene expression, in situ hybridization, immunoelectron microscopy

Introduction

Skin is composed of cells and extracellular matrix and serves as a protective barrier against environmental and infectious agents. Tissue injury disrupts this barrier, triggering a healing process involving a complex interplay of the cells and surrounding tissues [1]. The process includes consecutive phases of coagulation, inflammation, angiogenesis, matrix deposition, and tissue contraction. The synthesis of collagen and other extracellular molecules provide the matrix to fill the lost tissue.

Collagens are the major constituents of extracellular matrices [2,3]. Type V collagen is a minor component of connective tissues. Type V collagen is found in tissues where type I collagen is expressed. Type V collagen co-polymerizes with type I, to form the fibrils, and acts as a regulator of the size and the shape of the fibrils [4–6]. There are several type V isoforms that differ in chain composition. The major isoform is $[\alpha1(V)]_2\alpha2(V)$ which is present in many tissues. Defects in the human *COL5A1* and *COL5A2* genes have been identified in types I, II, and III Ehlers–Danlos syndrome [7,8].

An α1(V)α2(V)α3(V) heterotrimer has also been identified [9]. This isoform is observed in the human placenta as well as the uterus, skin, and synovial membranes [10–12]. The pro-α3(V) chain is also expressed in bone as well as in developing muscle and nascent ligament [12,13]. The N-terminal globular domain of the pro-α3 (V) chain has a unique structure that contains a highly basic peptide and influences bone formation by osteoblasts through proteoglycans on the cell surface [13].

Types I, III, and V collagens are expressed in experimental wound healing models [14]. There is lower expression of type V than that of types I and III. The α1(V) chain is expressed at day 5 and it is synthesized by fibroblast-like and rounded cells [15]. This study examined the expression of the α3(V) chain in wound healing tissues using in situ hybridization and immunohistochemistry.

Materials and Methods

Animals

The Imprinting Control Region mice and Wistar rats were purchased from commercial sources (Yoshitomi, Fukuoka, Japan). The animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Three and 10 mice were used in each group of reverse transcriptase polymerase chain reaction (RT-PCR) and morphological experiments, respectively.

Skin Wounding

Six-week-old mice were used for the experiment. They were anesthetized by intraperitoneal injection of the appropriate dose (5 μg/g weight) of sodium pentobarbital. The dorsum was shaved and cleaned with 70% ethanol, and full-thickness wounds were made aseptically on the dorsal skin with a disposable biopsy punch (5 mm diameter) [16,17]. The animals were killed on days 2, 4, 6, 10, and 15 after wounding.

Real-Time RT-PCR

Total RNA (5 μg) from a mouse skin was reverse transcribed using Maloney Murine Leukemia Virus reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA) with random hexamers at 37°C for 1 hr. The reaction sample was then heated at 95°C for 5 min to stop reaction. For a quantitative analysis of the expression level of the mRNA, real-time PCR was performed using a LightCycler

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TaqMan Master (Roche, Indianapolis, IN, USA). The thermal cycling conditions included 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and at 60°C for 1 min. The relative mRNA expression level of *Col1a1*, *Col3a1*, *Col5a1*, and *Col5a3* was normalized against that of the GAPDH gene from the same RNA preparations using a comparative threshold cycle method. For each gene, the relative expression value normalized to GAPDH before injury was 1.0. The primer sequences and the size of fragments are listed in Supplementary Table S1 (available online at http://informahealthcare.com/doi/suppl/[10.3109/03008207.2011.653061]).

Preparation of Sections

For in situ hybridization and immunohistochemistry, the tissues were cut from the lesion, fixed overnight in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS), washed in PBS (pH 7.4), and then dehydrated by passage through an ethanol series (50%, 70%, 80%, 95%, and twice × 100%). After embedding the tissues in a paraffin block, 10 μm consecutive sections were prepared.

In Situ Hybridization

cDNA clones for *Col1a1* (accession number: BC050014) and *Col3a1* (accession number: X52046) genes were isolated from a library of 17.5-day whole mouse embryo (Clonetech, Palo Alto, CA, USA). The cDNA for *Col5a1* and *Col5a3* genes was previously described [13]. The amplified fragments were subcloned into the pGEM-T Easy vector (Promega, Madison, WI, USA). The inserts were further subcloned into a pBluescript vector for generating riboprobes. All constructs were sequenced on an ABI310 sequencer (Applied Biosystems, Foster, CA, USA). After linearization at the appropriate restriction sites, antisense and sense probes were generated by in vitro transcription with T3 or T7 polymerases in the presence of [^{35}S]-dUTP. In situ hybridization was performed as previously described [13].

Antibodies

Mouse monoclonal antibodies against heparan sulfate and dermatan sulfate were purchased from commercial sources (Seikagaku, Tokyo, Japan).

Antibodies against pro-α3(V) and pro-α1(V) collagen chains were generated as previously described [13]. The fusion proteins glutathione S-transferase (GST)-tagged pro-α3(V) and pro-α1(V) were expressed and prepared according to the manufacturer's instructions. The recombinant GST-pro-α3(V) and GST-pro-α1 (V) collagens (0.5 mg) were mixed with Freund's Complete Adjuvant (Difco, Detroit, MI, USA) and subcutaneously injected into female Wistar rats. The animals were boosted twice with the same amount of protein mixed with Freund's Incomplete Adjuvant (Difco) and the blood was collected 1 week after the second booster. The antibody was purified using GST affinity column chromatography. The specificity of the antibodies was confirmed based on the distribution pattern of the pro-α3(V) chain using immunohistochemistry as previously described [13].

Immunohistochemistry

The tissues were fixed in 4% paraformaldehyde in 0.05M cacodylate buffer for 2 hr, dehydrated in a graded series of ethanol and embedded in paraffin. Consecutive 5 μm sections were cut. The sections were evaluated by indirect immunogold-silver staining [18]. Sections were incubated with the primary antibody for 2 hr at room temperature. The sections were rinsed in PBS and incubated with the gold-labeled secondary antibody (goat anti-rat IgG-gold, 5 nm; Amersham Biosciences, Buckinghamshire, UK), for 1 hr at room temperature. The sections were finally developed for 28 min at 23°C with the physical developer. (Solution A: 30 ml redistilled water, 12 ml 18-crown-6 (Sigma-Aldrich, St. Louis, MO, USA), 10% $AgNO_3$ aqueous solution 1 ml. Solution B: 30 ml 0.2M citrate buffer (pH 3.40–3.45), 100 mg hydroquinone.). The working developing solution was prepared by gently mixing solution A and solution B prior to use. The reaction products were black. The immunolabeled sections were observed and photographed.

Transmission Electron Microscopy

The tissues were immersion-fixed in twofold diluted Karnovsky's fixative for 2 hr at 4°C, then washed and immersed in 0.1M cacodylate buffer (pH 7.4) overnight at 4°C. The specimens were post-fixed in 2% osmium tetroxide–1% potassium ferrocyanide–0.05M cacodylate buffer (pH 7.4) for 2 hr at 4°C, dehydrated in an ascending series of ethanol, and finally embedded in epoxy resin. Ultrathin sections were cut on an ultramicrotome (LKB2088 Ultrotom V, Bromma, Sweden), mounted on copper grids, and stained with uranium acetate and lead citrate. The sections were observed and photographed with a transmission electron microscope (TEM; model 1200EX II; JEOL, Tokyo, Japan).

Immunogold Electron Microscopy

The tissues were fixed in a mixture of 3% paraformaldehyde and 0.1% glutaraldehyde in 0.05M cacodylate buffer (pH 7.4). Small pieces of tissues were dehydrated in an ascending series of ethanol and embedded in LR White Resin (London Resin Company, Reading, England). Ultrathin sections were cut on an ultramicrotome (LKB2088) and mounted on nickel grids. The nickel grids were placed directly on drops of 2% BSA (Sigma fraction V) in 0.01M phosphate buffer (pH7.4) containing 0.15M NaCl (PBS) to block nonspecific binding. Sections were then incubated with the primary antibody diluted 1:100 in PBS containing 0.1% BSA overnight in a humidified chamber at 4°C. The sections were thoroughly washed in the same PBS and bound antibodies were detected with 5 and 15 nm gold (Amersham Biosciences) at a final dilution of 1:100. Sections were rinsed in PBS and contrasted with uranium acetate followed by lead citrate. The sections were observed and photographed with the TEM.

Results

Expression of Pro-α3(V) Collagen Chain in Wound Healing

Real-time RT-PCR was initially performed using mouse skin tissue before and after injury to examine the expression of pro-α3(V) collagen chain. Pro-α3(V) chain expression was slightly detected before the injury and clearly increased on day 2 after the injury (Figure 1). The maximum expression was seen on day 4, and it gradually decreased thereafter and then returned to the level similar to that observed before the injury on day 20. The peak expression of the pro-α3(V) collagen chain was earlier than that of the pro-α1(V) and pro-α1(I) collagen chains, which peaked on day 10.

In situ hybridization was performed on the skin tissue after the injury to determine the precise expression of pro-α3(V) collagen chain (Figure 2). Weak signals were detected on day 2 and clearly seen on days 4 and 6 in the newly synthesized tissues of dermis. However, they were barely detected in the skin tissue on day 15 after the injury using the in situ hybridization method. Pro-α1(V) collagen chain signals were stronger than those of pro-α3(V) collagen chain in injured tissue after day 2. These signals were still strong on day 15. The pro-α1(I) and pro-α1(III) collagen chain signals were easily seen in normal connective tissue before

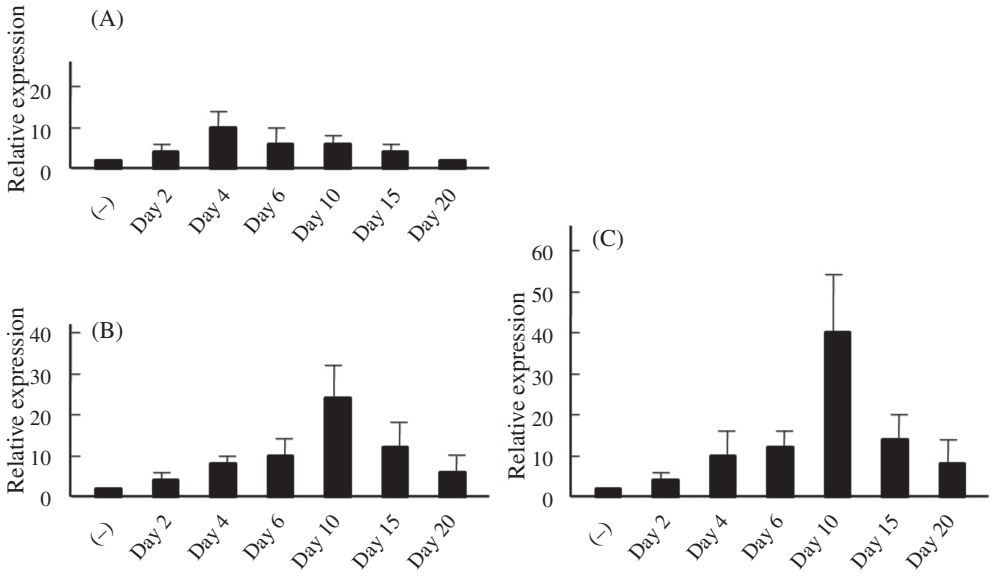

Figure 1. Real-time RT-PCR was performed to examine the amount of mRNA of pro-α3(V) (A), pro-α1(V) (B), and pro-α1(I) collagen gene (C) in wound healing tissues. RNA was isolated from the tissues before (–) and on days 2, 4, 6, 8, 10, 15, and 20 after injury. GAPDH was used to normalize the collagen gene expression levels. In each gene, the relative expression value against GAPDH before injury was 1.0. Data present the mean ± SD of at least three independent experiments.RT-PCR, real-time reverse transcriptase polymerase chain reaction.

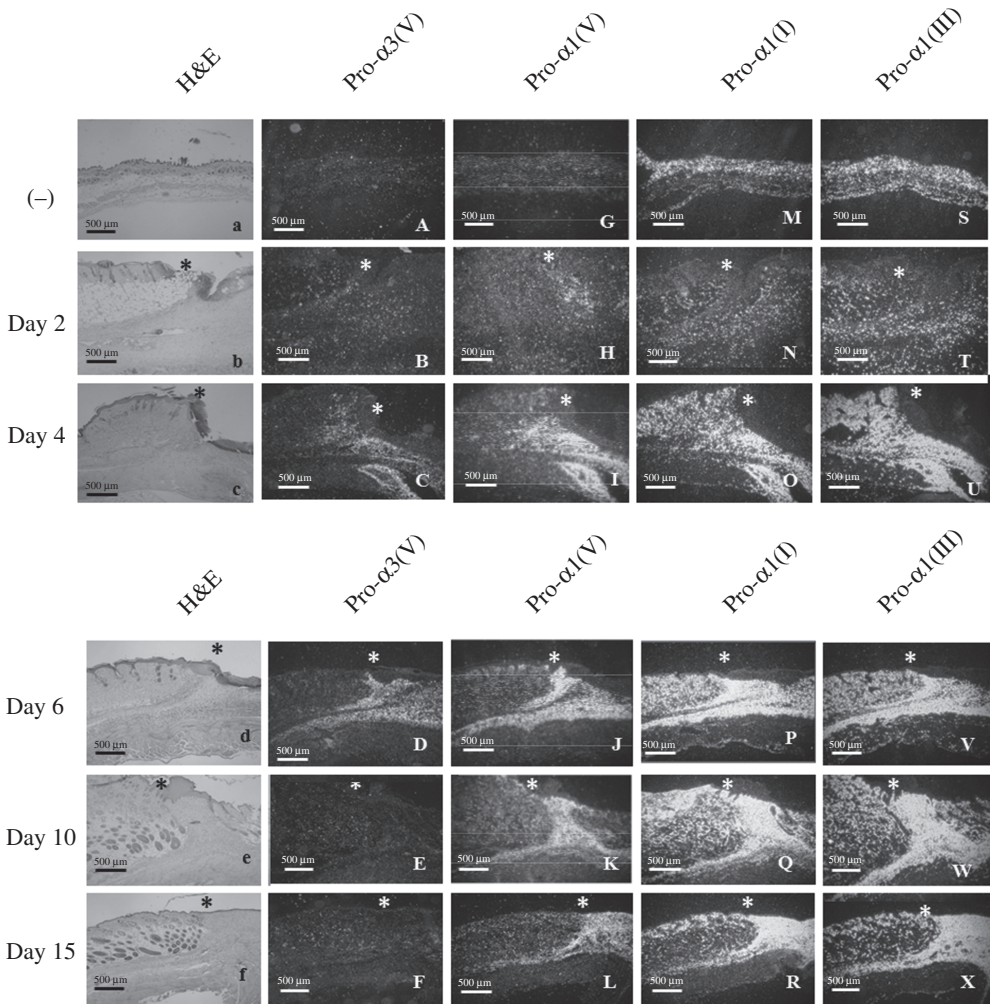

Figure 2. In situ hybridization of the wound healing tissues before (–) and on days 2, 4, 6, 10, and 15 after injury. The sections were hybridized with radioactively labeled pro-α3(V) (A–F), pro-α1(V) (G–L), pro-α1(I) (M–R), and pro-α1(III) collagens (S–X) antisense riboprobes. The figure shows darkfield photomicrographs. H&E staining is shown as control (a–f). Asterisks show the wound edges. Scale bar: 500 μm.
H&E, hematoxylin and eosin.

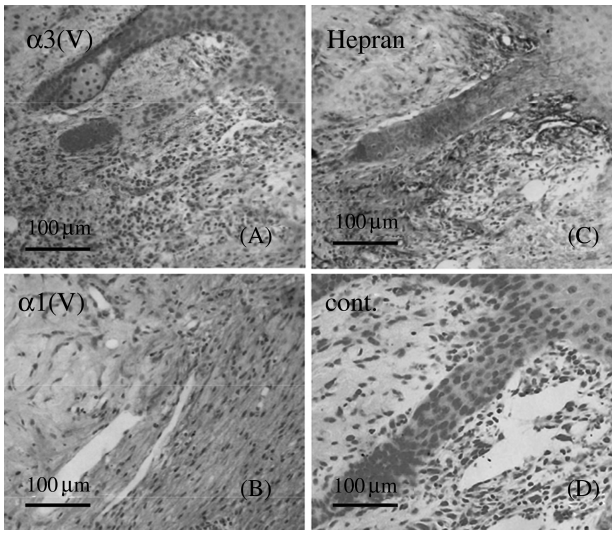

Figure 3. Immunohistochemical analysis of the wound healing tissues. The sections were immunostained with anti-pro-α3(V) (A) and anti-pro-α1(V) collagen chains (B), anti-heparan sulfate (C), and normal serum (D). The tissues are skin on day 6 after injury. Note that the reaction products are black. Scale bar: 100 μm.

the injury. These signals elevated dramatically on day 4 after the injury. These expression patterns were similar to those observed for real-time RT-PCR.

Distribution of Pro-α3(V) Collagen Chain in Wound Healing

Polyclonal antibody was prepared against pro-α3(V) chain to detect the protein. The specificity was confirmed based on the staining of pro-α3(V) chain in the umbilical cord where it was previously reported (Figure S1). The pro-α3(V) chain was seen on day 6 after the injury (Figure 3A). This finding was consistent with that of in situ hybridization (Figure 2D). The pro-α1(V) chain showed stronger signals in the injured region of dermis (Figure 3B). Heparan sulfate was strongly expressed on day 6 after the injury (Figure 3C). Dermatan sulfate was abundantly expressed in normal skin, but heparan sulfate was not (Figure S2).

Localization of Immunogold Staining

Electron microscopy was performed to examine the fine localization of pro-α3(V) chain. Light microscopy suggested that the pro-α3(V) chain was localized in the loose connective tissue rather than the dense connective tissue. The pro-α3(V) chain was localized in an amorphous nonfibrillar region, but not in the fibrils of the umbilical cord (Panel A in Figure S3). The small diameter fibrils and fibroblasts in wounded region were observed with electron microscope in the injured skin on day 6 (Figure 4A). The pro-α3(V) chain was seen in the amorphous nonfibrillar regions of the injured skin (Figure 4B), which was similar to those in the umbilical cord. On the other hand, pro-α1(V) chain was localized in the fine fibrils (Figure 4D) as well as in the amorphous nonfibrillar region (Figure 4C). Double staining was performed to examine the association with heparan sulfate. Heparan sulfate was seen in the amorphous nonfibrillar region in the umbilical cord (Panel B in Figure S3) and the wounded skin (Figure 4E) and the outer region of the basement membrane (Figure 4F). There were fewer pro-α3(V) chain signals co-localized in the amorphous nonfibrillar region and the basement membrane zone (Figure 4E and 4F).

Discussion

Pro-α3(V) collagen is expressed in newly synthesized bone as well as in the superficial fascia of developing muscle and the umbilical cord [12]. The current study examined the expression using an animal model of wound healing. The expression was transient and lower than that of other fibrillar collagen genes. The area of the expression was rather restricted. Electron microscopy demonstrated that pro-α3(V) collagen was localized in the amorphous nonfibrillar region.

Minor collagen regulates the diameter of collagen fibrils. The structure depends on the ratio of minor/major collagen and the species of chain [4–6]. The fibrils in cartilage consist of type II/XI collagen and form fine fibrils with a diameter of 20 nm [19]. On the other hand, type I/V collagen fibrils show diameters ranging from 30 to 150 nm (average, 90 nm) in 12-week-old mouse skin [20]. Type V collagen is required for collagen fibril nucleation [21]. The region containing the pro-α3(V) collagen chain was not localized in fibrils, while pro-α1(V) collagen was localized both in the fine fibrils and in the nonfibrillar region. Pro-α3(V) is only found in pro-α1(V)α2(V)α3(V) trimers [9]. Therefore, pro-α1(V)α2(V)α3(V) trimers might be located in the nonfibrillar region, but not in the fine fibrils.

Pro-α3(V) collagen has a unique domain that contains a highly basic segment of 23 amino acid sequence. The peptide has four repeats of the heparin-binding sequence BBXB (B and X are a basic and any amino acids, respectively), and bind to the heparin column with high affinity [13]. There is no heparan sulfate in the normal skin, but highly expressed after injury. The pro-α3(V) collagen signals co-localized with those of heparan sulfate, although there were fewer pro-α3(V) collagen signals. Heparan

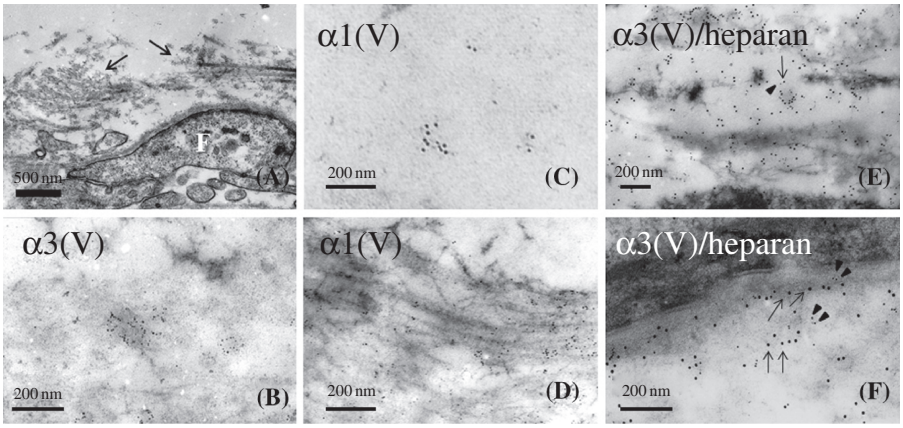

Figure 4. Immunohistochemical analysis using electron microscopy. The sections were immunostained with anti-pro-α3(V) (B), anti-pro-α1(V) collagen chains ((C) and (D)) and double-stained with anti-heparan sulfate (15 nm gold particles; arrows) and anti-pro-α3(V) collagen (5 nm gold particles; arrow heads) ((E) and (F)) antibodies. The tissue is skin on day 6 after injury. TEM analysis was also performed as a control (A). The small diameter fibrils and fibroblast are indicated with arrows and the letter F, respectively. Scale bar: 500 nm in (A) and 200 nm in ((B)–(F)).
TEM, transmission electron microscopy.

sulfate might bind to the pro-α3(V) collagen chain via this basic segment in the amorphous nonfibrillar region, and heparan sulfate is a side chain of perlecan that might also bind the pro-α3(V) collagen chain in the basement membrane zone.

The healing process is a complex process in the skin resulting in the formation of new connective tissue. Inkinen et al. [15] found that the localization of type V collagen was highly associated with blood vessels and granulation tissue in a wound healing model with subcutaneously implanted viscose cellulose sponges. The present study found that the pro-α3(V) and pro-α1(V) chains localized in the newly synthesized matrix. However, they did not seem to specifically accumulate around the capillaries, although they are observed to be present there. The pro-α3(V) chain was only expressed in the initial stage at which the fibrosis begins. The matrix containing the pro-α3(V) may therefore be needed for the initiation of tissue in pathological condition as well as of normal development in the tissues.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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