Treatment of liver ischemia–reperfusion injury by limiting thrombospondin-1/CD47 signaling

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Background. Ischemia–reperfusion (I/R) injury remains a primary complication of transplant surgery, accounting for about 80% of liver transplant failures, and is a major source of morbidity in other pathologic conditions. Activation of endothelium and inflammatory cell recruitment are central to the initiation and promulgation of I/R injury, which can be limited by the bioactive gas nitric oxide (NO). The discovery that thrombospondin-1 (TSP1), via CD47, limits NO signaling in vascular cells and ischemic injuries in vivo suggested that I/R injury could be another important target of this signaling pathway.

Methods. Wild-type, TSP1-null, and CD47-null mice underwent liver I/R injury. Wild-type animals were pretreated with CD47 or control antibodies before liver I/R injury. Tissue perfusion via laser Doppler imaging, serum enzymes, histology, and immunohistology were assessed.

Results. TSP1-null and CD47-null mice subjected to subtotal liver I/R injury showed improved perfusion relative to wild-type mice. Null mice subjected to liver I/R had decreased liver enzyme release and less histologic evidence of injury. Elevated TSP1 expression in liver tissue after I/R injury suggested that preventing its interaction with CD47 could be protective. Thus, pretreatment of wild-type mice using a blocking CD47 antibody improved recovery of tissue perfusion and preserved liver integrity after I/R injury.

Conclusions. Tissue survival and perfusion after liver I/R injury are limited by TSP1 and CD47. Targeting CD47 before I/R injury enhances tissue survival and perfusion in a model of liver I/R injury and suggests therapeutics for enhancing organ survival in transplantation surgery. (Surgery 2008;144:752-61.)

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Ischemia–reperfusion (I/R) injury is a complex process that involves a variety of pathophysiologic mechanisms. Upregulation of adhesion molecule expression mediates increased adhesion of lymphocytes and neutrophilic granulocytes to organ endothelium and their subsequent extravasation. These in turn release inflammatory cytokines and generate reactive oxygen species that mediate tissue damage. Total body or localized organ damage mediated by I/R injury is relevant in a variety of surgical fields such as transplantation medicine, cardiac surgery, and trauma surgery. Intervals of ischemia are also encountered during solid organ transplantation, myocardial revascularization, shock, and a variety of traumatic situations. Total and subtotal limb and acral part (ie, scalp, nose, eye lids, lips, ears, digits) amputations create periods of profound ischemia that initiate an I/R response. Microsurgical replantation of devascularized tissues and organs also initiates I/R injury.

The pathophysiology of liver I/R injury includes direct cellular damage as the result of the ischemic insult as well as delayed dysfunction and damage that results from activation of inflammatory pathways.1 Histopathologic changes include cellular swelling, vacuolization, endothelial cell disruption, neutrophil infiltration, and hepatocellular
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necrosis. The distal cascade of inflammatory responses that result in organ damage after I/R injury has been studied extensively. Activation of Kupffer cells with production of reactive oxygen species, upregulation of the inducible nitric oxide synthase and proinflammatory cytokines, and neutrophil accumulation contribute to inflammation-associated liver damage.

Nitric oxide (NO) is a constitutively produced bioactive gas with wide ranging physiologic properties. At low to moderate levels, NO promotes angiogenesis and tissue survival and directly increases blood flow and tissue perfusion. Inhibition of NO production worsened the outcome in a model of myocardial I/R injury. Conversely, administration of NO gas markedly improved the cardiac response to I/R injury. L-Arginine, the precursor for NO synthesis, added to cardioplegia solution dramatically reduced cardiac injury during cold storage. Therapies that increase either endogenous or exogenous NO are also beneficial in protecting other major organs from I/R injury. Alterations in serum liver enzymes were decreased in animals treated with L-arginine after liver I/R injury. Intestinal I/R injury after pretreatment with L-arginine was also reduced and was associated with enhanced wound healing. Treatment with NO donor compounds in murine models of liver I/R injury dramatically decreased hepatic necrosis. The precise role NO plays in the response of tissues to I/R injury depends on the nature of the organ system and injury.

Recently, we reported that the secreted matricellular protein thrombospondin-1 (TSP1) potently blocks NO/cyclic 5′-guanosine monophosphate signaling in vascular endothelial cells, vascular smooth muscle cells, and platelets, and that this process requires interaction with the cell-surface receptor CD47. The physiologic implications of this are many. Deletion of TSP1 or CD47 in transgenic mice dramatically increases blood flow after NO challenge and enhances soft tissue and hindlimb survival of fixed ischemia. Blocking the TSP1–CD47 signal in wild-type mice and pigs similarly confers increased ischemic tissue survival, blood flow, and perfusion. These therapeutic advantages were demonstrated using in vivo models of subtotal and total fixed ischemia. It is not clear, however, whether TSP1 signaling through CD47 has a similar effect on reperfusion injury. In the present report, we demonstrate enhanced reperfusion and decreased tissue damage after I/R injury in the absence of TSP1 and CD47. We further show that an antibody targeting CD47 can mitigate the complications of liver I/R injury in wild-type mice.

METHODS

Animals. Wild-type, TSP1-null, and CD47-null C57BL/6 mice were housed under pathogen-free conditions and had ad libitum access to filtered water and standard rat chow. Handling and care of animals was in compliance with the guidelines established by the Animal Care and Use Committees of the National Cancer Institute.

Liver I/R model. Age- and sex-matched wild-type, TSP1-null, and CD47-null mice were maintained with 1.5% isoflurane anesthesia. Using loupe magnification and sterile technique, the right and left upper quadrants of the abdomen were entered via a chevron incision, the branches of the portal triad to the left/median lobes of the liver were identified and a 1-g force microsurgical clamp applied for an ischemic interval of 45 minutes. This approach, commonly referred to as a subtotal I/R liver injury model, does not induce complete intestinal outflow obstruction as found with occlusion of the vascular flow to all portions of the liver. Before triad occlusion, diaphragmatic attachments to the liver were incised allowing for anterior and inferior migration of the liver and minimizing motion artifact on laser Doppler analysis from respiratory activity. Animal core temperature was continuously monitored via rectal probe and maintained at 35.5°C with a warming surface and heat.
Evaporative loss was minimized with sterile plastic wrap applied to the anterior abdominal wall wound. Animals experienced either 60 or 360 minutes of reperfusion. In those animals undergoing 360 minutes of reperfusion, the anterior abdominal wall was closed in a layered manner after clamp removal with interrupted 5-0 nylon suture. Animals recovered and, when mobile, were returned to their cages. At the end of the reperfusion interval, animals were again anesthetized with isoflurane, the anterior abdominal wall closure opened, and the liver exposed to allow for laser Doppler analysis. In another group of control wild-type, TSP1-null, and CD47-null animals, sham operation without I/R injury was performed. Liver blood flow before and after I/R injury was determined via laser Doppler at the indicated time intervals. Serum and tissue samples were processed for enzyme levels, histology, and immunohistology as described. Data represent results of studies performed in a total of 102 animals distributed as follows: untreated liver I/R group (n = 48) including wild type (n = 24); TSP1 null (n = 12); CD47 null (n = 12); treated liver I/R (n = 30), 15 receiving a CD47 antibody and 15 receiving an isotype-matched control antibody; and sham groups, (n = 24), 8 each of wild type, TSP1 null, and CD47 null.

**CD47 antibody treatment.** Age- and sex-matched wild-type C57BL/6 mice were randomized in 2 experimental groups and received either a rat anti-mouse CD47 monoclonal antibody (clone 301; 0.4 mg/g weight IP in 100 μL sterile phosphate-buffered saline [PBS]) 90 minutes before surgery or a similar dose of an immunoglobulin (Ig)G2a isotype-matched antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) in 100 μL of sterile PBS. Liver blood flow before and after I/R injury was determined via laser Doppler at the indicated time intervals. Serum and tissue samples were processed for enzyme levels, histology, and immunohistology as described.

**Laser Doppler analysis.** Liver blood flow was measured using laser Doppler imaging (MoorLD1-2; Moor Instruments, Devon, UK). Briefly, animals were placed in a supine position on a heating pad, and anesthesia was provided by 1.5% inhalation isoflurane in a 50:50 mixture of oxygen to room air. Core temperature was maintained via heat lamp at 35.5°C and monitored by rectal probe. The hair of the ventral surface of the anterior abdominal wall or respective hindlimb was clipped and depilated with Nair and a region marked. After equilibration to the experimental setup, analysis of baseline hepatic blood flow was obtained. The following instrument settings were used: override distance, 21 cm; scan time, 4 msec/pixel. Results are expressed as the percent change from baseline control of the region of interest.

**Serum analysis of liver function.** Blood was obtained from animals following I/R injury with a 1-mL heparin-wetted syringe via direct cardiac puncture. Blood was collected in heparin-coated syringes, centrifuged, serum separated, and immediately processed for serum and liver enzymes. Results represent the mean values ± SD of 18 animals, 6 of each genotype. *P < .05 CD47 null sALT versus wild type indicated by * and TSP1 null and CD47 null serum aspartate aminotransferase null versus wild type indicated by ** (A) or TSP1 null and CD47 null sALT and sALT versus wild type indicated by * (B).

**Fig 2.** Serum enzyme markers are reduced in null animals after acute and subacute reperfusion. After liver ischemia and 60 (A) or 360 (B) minutes of reperfusion as described and while maintained at a constant core temperature and under isoflurane anesthesia sex- and age-matched wild-type, TSP1-null, and CD47-null mice underwent direct cardiac puncture. Blood was collected in heparin-coated syringes, centrifuged, serum separated, and immediately processed for serum and liver enzymes. Results represent the mean values ± SD of 18 animals, 6 of each genotype. *P < .05 CD47 null sALT versus wild type indicated by * and TSP1 null and CD47 null serum aspartate aminotransferase null versus wild type indicated by ** (A) or TSP1 null and CD47 null sALT and sALT versus wild type indicated by * (B).
Fig 3. TSP1-null and CD47-null liver sections demonstrate minimal damage after I/R injury. After 45 minutes of liver ischemia and either 60 or 360 minutes of reperfusion, age- and sex-matched wild-type, TSP1-null, and CD47-null mice were killed and the left and middle hepatic lobes excised, processed, and stained with hematoxylin-eosin (A; original magnification, ×10). Liver sections from sham operated animals were treated similarly. Images are representative liver sections from 18 mice, 6 of each strain. Inflammatory cell infiltrate was determined in 10 high-power fields from wild-type, TSP1-null, and CD47-null sections (B). Results are from analysis of 72 total sections, 4 sections each from 18 liver lobes, 6 of each genotype. After 45 minutes of ischemia and 360 minutes of reperfusion, wild-type, TSP1-null, and CD47-null liver sections were stained for evidence of tissue apoptosis/necrosis (C; original magnification, ×20). Images are representative of sections from 6 mice of each genotype.
Fig 4. Monoclonal antibody targeting of CD47 decreases I/R liver damage. Age- and sex-matched wild-type C57BL/6 mice received either a monoclonal CD47 antibody (clone 301) or an isotype-matched control IgG2a antibody 90 minutes preoperatively. Laser Doppler analysis of liver tissue perfusion was performed preoperatively, after 45 minutes of ischemia, and after 360 minutes of reperfusion (A). Results represent the mean values ± SD of 30 animals, 15 in each treatment group. Blood was collected for analysis of serum liver enzymes (B). Results represent the mean values ± SE of 12 animals, 6 in each treatment group. *P < .05 IgG2a versus 301. Liver tissue sections were processed for immunohistology. Normal and post-I/R injury liver sections (360 minutes reperfusion) from wild-type and CD47-null animals (C) (original magnification, ×20) or liver sections from wild-type animals pretreated with a CD47 antibody (301) or an isotype control IgG2a before I/R injury (360 minutes reperfusion; (D) were stained with a monoclonal TSP1 antibody (clone A6.1; original magnification, ×10). Inflammatory cell infiltrate was determined in 10 high-power fields from wild-type, TSP1-null, and CD47-null sections (E). Results are from analysis of 48 total sections, 4 sections each from 12 liver lobes, 6 animals in each group.
puncture, centrifuged at 4°C, and serum collected and immediately analyzed. To assess hepatic function and cellular injury after liver ischemia serum enzymes including alanine aminotransferase (sALT) and aspartate aminotransferase were measured using the Synchron LX 20 System Chemistry Analyzer (Beckman Coulter, Fullerton, Calif) by the Department of Laboratory Medicine, Clinical Center, National Institutes of Health.

**Histology.** Hepatic tissues (ischemic and normal lobes) were fixed in 10% formalin, embedded in paraffin; 5-μm-thick sections cut and stained with hematoxylin-eosin, and examined by a pathologist. Leukocyte infiltration was evaluated to determine the severity of inflammation. Each liver section was divided into 10 subsections, and polymorphonuclear cell infiltration was examined by a trained pathologist blind to strain or treatment administered in each of subsections at a magnification of ×400 with cells found in sinusoids or tissue counted and those in hepatic vessels disregarded.

**Immunohistochemistry.** Paraffin-embedded livers were sectioned at a thickness of 5 μm and applied to charged glass slides and processed for immunohistology. Briefly tissue sections were deparaffinized with xylene and rehydrated in alcohol. Slides were then heat inactivated in 10 mmol/L sodium citrate (pH 6.0) in a microwave for 5 minutes. Cooled slides were rinsed with PBS and then incubated with 3% H₂O₂ for 30 minutes at room temperature. Sections were then blocked with 5% normal goat serum in PBS for 30 minutes at room temperature followed by a 12-hour incubation in a humidified chamber at 37°C with a rat anti-murine macrophage antibody CD68 (clone FA-11; AbD Serotec, Oxford, UK) at a 1:50 dilution, or a TSP1 monoclonal antibody clone A6.1 (LabVision, Fremont, Calif) at a 1:100 dilution. Slides were washed and then incubated with goat anti-rabbit IgG-biotin conjugate (BD PharMingen, Franklin Lakes, NJ) diluted at 1:100 with PBS and incubated in prediluted streptavidin–horse radish peroxidase conjugate (BD PharMingen) for 45 minutes at room temperature. Color was developed by DAB substrate kit (BD PharMingen). Slides were counterstained using Mayer’s hematoxylin for 2 minutes, dehydrated, and mounted.

**Programmed cell death.** The ApopTag in situ detection kit (Chemicon; Millipore, Billerica, Mass) was employed following the manufacturer’s recommendations. In brief, sections underwent deparaffinization, rehydration, and washing, followed by treatment with 20 μg/mL of proteinase K for 15 minutes at room temperature and repeat washing. Endogenous peroxidase activity was quenched with 3% H₂O₂ in PBS for 5 minutes. The 3'–hydroxy DNA strand breaks were enzymatically labeled with digoxygenin nucleotide via TdT and incubated for 1 hour at 37°C, and the reaction terminated with a stop buffer. Sections were then incubated with anti-digoxygenin peroxidase for 30 minutes at room temperature, washed, stained with 3-3’-diaminobenzidine substrate, counterstained with methyl green, and mounted. Positive and negative control slides provided with the kit were used in each assay to ensure consistency.

**Statistics.** Results are presented as the mean values ± SD or where indicated as the mean values ± SE with significance calculated by the Student’s t test or ANOVA. Significance was assigned a P value of ≤ .05.

**RESULTS**

TSP1 and CD47 limit acute reperfusion after liver I/R injury. Wild-type and null animals underwent a comparable degree of liver I/R injury as documented by laser Doppler analysis of blood flow in all groups. However, flow after 60 minutes of reperfusion was significantly greater in the TSP1 null and CD47 null animals (Fig 1, A).

TSP1 and CD47 limit subacute reperfusion flux after liver I/R injury. We next extended the reperfusion time to 360 minutes. Despite comparable degrees of temporary ischemia in all groups, laser Doppler analysis of blood flow demonstrated that liver reperfusion was significantly greater after 360 minutes of reperfusion in CD47 and TSP1 null animals than in the wild type controls (Fig 1, B, C).

Liver I/R injury is decreased in the absence of TSP1 and CD47. After liver I/R injury and both 60 and 360 minutes of reperfusion we assessed liver enzyme levels. Serum ALT was significantly lower in CD47-null animals and serum AST significantly lower in both TSP1-null and CD47-null animals compared with wild-type as early as 60 minutes after clamp removal and flow restoration (Fig 2, A). With extended reperfusion of 360 minutes, decreased serum levels of AST and ALT became significant for both CD47-null and TSP1-null animals (Fig 2, B).

The absence of TSP1 or CD47 preserves normal liver cyto-architecture after I/R injury. Hematoxylin and eosin staining of normal and ischemic liver was performed in wild-type and null animals. Wild-type ischemic liver sections demonstrated significant changes in histology at both 60 and 360 minutes of reperfusion with perilobular swelling, cell vacuolation, necrosis of hepatocytes, and destruction of parenchymal chords (Fig 3, A). In
contrast, TSP1-null and CD47-null ischemic livers demonstrated preservation of normal hepatic cytoarchitecture and showed markedly less necrotic areas, cell swelling, and vacuolation.

**Inflammatory infiltration of hepatic tissue is markedly reduced in TSP1-null and CD47-null animals after I/R injury.** Evidence of inflammatory cell infiltration was found in all ischemic liver sections after 360 minutes of reperfusion regardless of strain. However, leukocyte counts were significantly reduced in TSP1-null and CD47-null livers (Fig 3, B). Immunohistochemical staining of normal and ischemic liver for intrahepatic macrophages (CD68) was performed. Occasional CD68-positive cells were located in sections of nonischemic liver from all animals, although after 360 minutes of reperfusion minimal increase in macrophage infiltration was found in ischemic liver sections regardless of strain (data not shown).

**Hepatocyte programmed cell death after I/R injury is decreased in the absence of TSP1 and CD47.** Apoptosis and necrosis, as manifestations of programmed cell death, have been reported as a major feature of liver I/R injury. Analysis of tissue sections from wild-type, TSP1-null, and CD47-null livers after 6 hours of reperfusion demonstrated minimal to no programmed cell death in null sections (brown nuclear staining; Fig 3, C). In contrast, wild-type sections had substantial numbers of apoptotic and necrotic cells.

**Pretreatment with a CD47 antibody protects against liver I/R injury.** To investigate the potential for targeting CD47 to protect liver from damage secondary to I/R injury, a rat anti-mouse CD47 antibody (clone 301) with demonstrated protective effects in a soft tissue model of fixed ischemia was administered to age- and sex-matched wild-type mice 90 minutes before ischemia. Other age- and sex-matched wild-type animals received a comparable dose of an isotype-matched IgG2a control antibody 90 minutes before ischemia. Ig antibodies persist for extended intervals in vivo with the major IgG subclasses lasting up to 3 weeks in people. Laser Doppler analysis of tissue perfusion after 360 minutes of reperfusion demonstrated significantly more robust blood flow in animals pretreated with the CD47 targeting antibody versus the isotype matched control (Fig 4, A; P < .05). Serum ALT and AST levels in animals receiving CD47 antibody were also significantly decreased relative to levels obtained from animals treated with the isotype-matched control IgG (Fig 4, B).

Antibody 301 is known to block CD47-dependent responses to TSP1 in fixed ischemic injuries and full-thickness skin grafts; therefore, we wanted to confirm that TSP1 is present in the liver to engage CD47 after I/R injury. TSP1 expression was minimal in livers of control (sham surgery) wild-type mice, but was markedly increased after I/R injury (Fig 4, C). After I/R injury, TSP1 expression was qualitatively lower in liver sections from CD47-null animals than in wild-type animals, although basal TSP1 expression was somewhat higher in the CD47-null livers from sham operated animals (Fig 4, C). After I/R injury, TSP1 staining in the liver was markedly less in wild-type animals treated with the CD47 targeting antibody (clone 301) versus the control antibody (Fig 4, D).

Liber necrosis, perilobular swelling, and inflammatory cell infiltration were also decreased after pretreatment with a CD47 antibody compared with controls treated with the isotype-matched IgG2a antibody (data not shown). These findings correlated with both decreased TSP1 expression and inflammatory cell infiltration in post-I/R injury sections from mice pretreated with a CD47 monoclonal antibody compared with sections from animals treated with the control antibody (Fig 4, D, E).

**DISCUSSION**

We demonstrate here a limiting role for TSP1 and CD47 in liver I/R injury. TSP1-null and CD47-null animals show markedly enhanced flow responses after I/R injury in a standard liver model. The absence of either protein is associated with significant increases in flow immediately and after 1 and 6 hours of reperfusion. TSP1 expression increases after I/R injury. Blocking TSP1 signaling via CD47 using a monoclonal antibody recognizing CD47 significantly enhances tissue reperfusion after I/R injury, essentially converting the wild-type phenotype to that of the null.

The protective role of blocking TSP1/CD47 signaling in liver I/R injury response may involve several mechanisms. TSP1/CD47 interactions are known to increase platelet activation, aggregation, and thrombosis in inflamed vasculature. Because TSP1/CD47 signaling blocks the antithrombotic activity of NO, enhanced flow dynamics in null animals after I/R injury may arise in part by augmenting the antithrombotic activity of NO.

Programmed cell death, manifesting as either cell necrosis or apoptosis, is a second important component of I/R injury. Cultured kidney cells demonstrated apoptotic changes after treatment with exogenous TSP1. Thyroid cells are protected from apoptotic cell death by a TSP1 peptide that binds to CD47 and both a gene silencing CD47 morpholino and a CD47 monoclonal antibody increased ischemic tissue survival.
Conversely, TSP1–CD47 interactions have been found to suppress non–I/R-driven inflammation induced by topical application of oxazolone owing to increased apoptosis of inflammatory cells. Compared with wild type, TSP1-null and CD47-null liver sections demonstrated dramatically less necrosis/apoptosis after I/R injury and 6 hours of reperfusion.

Recruitment of inflammatory leukocytes is another hallmark of I/R injury. Both TSP1 and CD47 are known to support the recruitment of inflammatory neutrophils, monocytes, and T cells. However, this is the first report of a specific role of CD47 in I/R injury. A CD47 agonist peptide sequence from the C-terminal domain of TSP1 increases monocyte adhesion to endothelial cells, whereas a CD47 monoclonal antibody (B6H12) blocks neutrophil transendothelial migration. Additionally, CD47 expression levels on endothelium correlated with increased neutrophil transmigration, and a monoclonal antibody to CD47 decreased PMN transmigration across intestinal epithelial layers in vitro. Therefore, the decreased inflammatory cell infiltration we documented in TSP1-null and CD47-null liver sections after I/R injury compared with wild type could reflect their specific requirement for leukocyte recruitment in response to I/R stimuli. Importantly, inflammatory cell infiltration after I/R injury in wild-type livers from animals pretreated with a CD47 antibody (clone 301) was substantially less compared with sections from animals pretreated with a matched IgG2a control antibody. Therefore, blocking CD47 may limit inflammatory cell recruitment via activated endothelium, and the absence of TSP1 may eliminate an important chemoattractant that mediates leukocyte recruitment to sites of I/R injury. Also, blocking TSP1–CD47 signaling has been found to decrease the generation of T-regulatory cells. The long-term suppression of TSP1–CD47 signaling could potentially decrease tolerance development to transplanted organs and would need to be considered in designing therapeutics targeting this pathway.

Our data showing increased TSP1 expression after I/R injury in the liver are consistent with several previous studies in other I/R injury models. In a rat model of middle cerebral artery I/R injury, TSP1 expression peaked at 1 hour and again at 72 hours. I/R injury in myocardial tissue was associated with increased TSP1 mRNA expression. TSP1 expression was increased in areas of cell apoptosis in a model of kidney I/R injury. Thus, elevated TSP1 expression is a common finding in tissue subjected to I/R injury and may be associated with increased programmed cell death of damaged cells. The mechanism for induction of TSP1 in I/R injury remains to be determined, but the known induction of TSP1 by hypoxic signaling and by changes in NO levels should be considered. Regarding the latter pathway, it is interesting that our immunohistochemical studies demonstrated lower TSP1 expression in liver sections after I/R injury in CD47-null mice. This implies that CD47 is both a target of TSP1 and an important mediator of the signals that control TSP1 expression.

These results suggest that therapeutic targeting of TSP or CD47 may enhance tissue survival after I/R injury. The applications of such therapeutics would include any ischemic injury that could be treated with agents to block TSP1 interactions with CD47 or lower their expression before reperfusion. Specific applications would include preservation of whole organs for transplantation, microsurgery and reimplantation, prevention of I/R injury after open heart surgeries, and stroke. It remains to be determined whether anti-CD47 therapeutics will also be beneficial when used post I/R injury.

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