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Glycophorin B is the erythrocyte receptor of *Plasmodium falciparum* erythrocyte-binding ligand, EBL-1

D. C. Ghislaine Mayera,1, Joann Cofiea, Lubin Jiangb, Daniel L. Hartlc, Erin Tracya, Juraj Kabad, Laurence H. Mendozaa, and Louis H. Millera,1

*Department of Biology, Virginia Commonwealth University, 1000 West Cary Street, Room 126, Richmond, VA 23284-2012; 2Laboratory of Malaria Vector Research, 12735 Twinbrook Parkway, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852; 3Biological Imaging Section, Research Technologies Branch, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 4 Center Drive, MSC 0485, Bethesda, MD 20892; and 4Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138*

Contribution by Louis H. Miller, January 29, 2009 (sent for review December 19, 2008)

In the war against *Plasmodium*, humans have evolved to eliminate or modify proteins on the erythrocyte surface that serve as receptors for parasite invasion, such as the Duffy blood group, a receptor for *Plasmodium vivax*, and the Gerbich-negative modification of glycophorin C for *Plasmodium falciparum*. In turn, the parasite counters with expansion and diversification of ligand families. The high degree of polymorphism in glycophorin B found in malaria-endemic regions suggests that it also may be a receptor for *Plasmodium*, but, to date, none has been identified. We provide evidence from erythrocyte-binding that glycophorin B is a receptor for the *P. falciparum* protein EBL-1, a member of the Duffy-binding-like erythrocyte-binding protein (DBL-EBP) receptor family. The erythrocyte-binding domain, region 2 of EBL-1, expressed on CHO-K1 cells, bound glycophorin B+ but not glycophorin B-null erythrocytes. In addition, glycophorin B+ but not glycophorin B-null erythrocytes adsorbed native EBL-1 from the *P. falciparum* culture supernatant. Interestingly, the Efe pygmies of the Ituri forest in the Democratic Republic of the Congo have the highest gene frequency of glycophorin B-null in the world, raising the possibility that the DBL-EBP family may have expanded in response to the high frequency of glycophorin B-null in the population.

Duffy-binding-like erythrocyte-binding protein | invasion | malaria | red blood cells

The asexual erythrocytic phase of the life cycle of *Plasmodium falciparum* produces all of the clinical symptoms, disease, and pathology associated with malaria. During this phase, merozoites released from schizont-infected erythrocytes invade uninfected erythrocytes. Invasion depends on distinct molecular interactions between ligands on the merozoite, the invasive form of the parasite, and host receptors on the erythrocyte membrane. To avoid infection, humans have evolved to eliminate or modify erythrocyte surface proteins that serve as receptors for parasite invasion. Perhaps one of the best examples of this evolutionary process is the loss of the Duffy blood group in Africa. *Plasmodium vivax* depends on two ligands for erythrocyte invasion: the Duffy-binding protein (DBP) that binds the Duffy blood group antigen (1, 2) and the reticulocyte homology protein that binds to an unknown receptor on reticulocytes (3). The Duffy blood group-null went almost to fixation in West Africa and is being selected in Papua New Guinea as a result of the inability of *P. vivax* to invade Duffy-negative erythrocytes (4).

Unlike *P. vivax*, *P. falciparum* has highly redundant, alternate invasion pathways that use several different receptor families. For example, *P. vivax* has only one gene, DBP, in the Duffy-binding-like erythrocyte-binding protein (DBL-EBP) family, whereas *P. falciparum* has four DBL-EBP genes: erythrocyte-binding antigen 175 (EBA-175), erythrocyte-binding antigen 140 (BAEEL/EB-140), erythrocyte-binding antigen 181 (JESEBL/ EBA-181), and erythrocyte-binding ligand-1 (EBL-1) (5–10). Consequently, no erythrocyte has been identified that is refractory to *P. falciparum* invasion. The erythrocyte-binding domains for *P. vivax* DBL-EBP and for three of the *P. falciparum* DBL-EBPs reside in the N-terminal cysteine-rich region, region 2 (8, 11, 12). In *P. falciparum*, the region 2 domain is duplicated; both domains are required for optimum binding to erythrocytes. The erythrocyte receptors for the *P. falciparum* DBL-EBPs include glycophorin A for EBA-175 and glycophorin C for one of the BAEEL/EB-140 proteins (6, 12). The receptor for JESEBL/EB-181 is not known. No *P. falciparum* ligand has been found to bind glycophorin B, even though glycophorin B is highly polymorphic, suggesting that it is under a strong selective pressure. The polymorphisms are especially high in Africans and in African–Americans where multiple mutations exist, including the S-s-U-blood group (glycophorin B-null) that has a gene frequency of 59% in the Ituri forest pygmies (13, 14).

Here, we provide evidence that the fourth DBL-EBP family member, EBL-1, binds to glycophorin B. Region 2 of EBL-1 expressed on CHO-K1 cells bound to normal erythrocytes but not to glycophorin B-null erythrocytes. In addition, EBL-1 immunoprecipitated from *P. falciparum* culture supernatant bound to normal erythrocytes but not to glycophorin B-null erythrocytes. EBL-1 also fails to bind chymotrypsin- and neuraminidase-treated erythrocytes, consistent with the erythrocyte receptor being glycophorin B, a protein that is sensitive to neuraminidase and chymotrypsin. Thus, these studies identify glycophorin B as the erythrocyte receptor of EBL-1.

**Results**

**Localization of EBL-1.** The spatial and temporal expression of EBL-1 has not been reported. To verify that the location of EBL-1 is similar to that of other members of the DBL-EBP family, we localized EBL-1 by fluorescent confocal microscopy. Antibodies specific for region 2 of EBL-1 from the *P. falciparum* culture supernatant bound to normal erythrocytes but not to S-s-U-erythrocytes. EBL-1 also fails to bind to neuraminidase-treated erythrocytes, consistent with the erythrocyte receptor being glycophorin B, a protein that is sensitive to neuraminidase and chymotrypsin. Thus, these studies identify glycophorin B as the erythrocyte receptor of EBL-1.

**Data deposition:** The sequences reported in this paper have been deposited in the GenBank database (accession no. FJ392548).
was it observed with the preimmune sera (Fig. S1).

specific for region 2 of EBL-1 contained a protein of kDa, the predicted size of EBL-1 (Fig. 2).

noprecipitation of the Dd2/Nm supernatant with antibodies specific for region 2 of EBA-175 contained a protein of 200 kDa. The antibodies specific for EBL-1 failed to immunoprecipitate a 300-kDa protein from the EBL-1-null clone HB3 (Fig. 2B) whereas an antibody to EBA-175 immunoprecipitated a protein of the correct size. To determine whether EBL-1 bound to erythrocytes, culture supernatant was incubated with ~50 μL of packed erythrocytes. The supernatant after absorption with human erythrocytes no longer contained EBL-1 and had a reduced amount of EBA-175, respectively. Treatment of the adsorbed erythrocytes with 1.5 M NaCl released both EBL-1 and EBA-175 from the erythrocytes, respectively (Fig. 2A).

**Glycophorin B is the Erythrocyte Receptor of EBL-1.** To characterize further the binding specificity of EBL-1, we studied binding to neuraminidase-, chymotrypsin-, and trypsin-treated human erythrocytes and human erythrocytes with genetically modified blood groups. Treatment with neuraminidase or chymotrypsin eliminated the ability of EBL-1 to bind human erythrocytes (Fig. 3A). However, EBL-1 bound to trypsin-treated erythrocytes in the same manner as untreated erythrocytes, indicating that the erythrocyte receptor required sialic acid attached to a peptide backbone that was susceptible to chymotrypsin but not to trypsin (Fig. 3A). One such protein on the erythrocyte with these characteristics is glycophorin B.

To define further the binding specificity of EBL-1, we used S-s-U- (glycophorin B-null) human erythrocytes from two different donors. S-s-U- erythrocytes from both donors did not bind EBL-1 from the lysates of the Dd2/Nm clone of *P. falciparum* (Fig. 4A). In contrast, EBA-175, the ligand for glycophorin A, bound to S-s-U- erythrocytes (Fig. 4B).

**Expression of EBL-1.** To determine the temporal expression profile of EBL-1 during the intraerythrocytic stage, protein extracts from rings, trophozoites, and schizonts of *P. falciparum* clone Dd2/Nm were used for immunoblotting with antibodies specific for region 2 of EBL-1. We found that EBL-1 is expressed only in the schizont stage of the intraerythrocytic life cycle. The expression profile is similar to that of other DBL-EBPs. In control experiments, EBL-1 was not observed in protein extracts from *P. falciparum* clone HB3 that lacks the *ebl-1* gene (9), nor was it observed with the preimmune sera (Fig. S1).

**Binding Characteristics of EBL-1.** To measure the binding of EBL-1 to human erythrocytes, we performed erythrocyte-binding assays by using soluble, metabolically labeled *P. falciparum* proteins. Schizont-infected erythrocytes were cultured without uninfected erythrocytes so that released merozoites would remain in the supernatant. Under such conditions, erythrocyte-binding proteins of the DBL-EBP family have been identified in the culture supernatants. Immunoprecipitation of culture supernatant of Dd2/Nm merozoites with antibodies specific for region 2 of EBL-1 contained a protein of ~300 kDa, the predicted size of EBL-1 (Fig. 2A). Similarly, immunoprecipitation of the Dd2/Nm supernatant with antibodies specific for region 2 of EBA-175 contained a protein of ~200 kDa. The antibodies specific for EBL-1 failed to immunoprecipitate a 300-kDa protein from the EBL-1-null clone HB3 (Fig. 2B) whereas an antibody to EBA-175 immunoprecipitated a protein of the correct size. To determine whether EBL-1 bound to erythrocytes, culture supernatant was incubated with ~50 μL of packed erythrocytes. The supernatant after absorption with human erythrocytes no longer contained EBL-1 and had a reduced amount of EBA-175, respectively. Treatment of the adsorbed erythrocytes with 1.5 M NaCl released both EBL-1 and EBA-175 from the erythrocytes, respectively (Fig. 2A).

**Fig. 1.** EBL-1 is located in the apical end of *P. falciparum* merozoites. Confocal microscopy of fully mature schizonts containing individual merozoites demonstrates the localization of EBL-1 in the apical end of the merozoite. (A) Dd2/Nm schizonts were double-labeled with rabbit anti-EBL-1 region 2 and an anti-BAEBL/EBA-140 polyclonal antibody. Schizonts immunolabeled with anti-EBL-1 region 2 were stained with Alexa Fluor 594 secondary antibody (red). Schizonts labeled with anti-BAEBL/EBA-140 were stained with Alexa Fluor 488 secondary antibody (green). (B) Dd2/Nm schizonts were double-labeled with anti-EBL-1 region 2 and anti-RAP1. Schizonts immunolabeled with anti-EBL-1 region 2 were stained with Alexa Fluor 594 secondary antibody (red). Schizonts labeled with anti-RAP1 were stained with Alexa Fluor 488 secondary antibody (green). (Scale bar, 10 μm.)

**Fig. 2.** EBL-1 binds to human erythrocytes. *P. falciparum* cultures from Dd2/Nm (A) and HB3 (B) were absorbed to human erythrocytes. EBL-1 was found in the culture supernatant (Unabs, unabsorbed; Abs, absorbed by erythrocytes) and eluate of Dd2/Nm but not in HB3. EBA-175 was immunoprecipitated from the culture supernatants and eluates of both.

**Fig. 3.** Erythrocytes treated with neuraminidase or chymotrypsin do not bind to EBL-1. (A) Immunoprecipitation with anti-EBL-1. (B) Immunoprecipitation with anti-EBA-175. Unabs, unabsorbed; Abs, absorbed with untreated or enzyme treated erythrocytes; U, untreated erythrocytes; NM, neuraminidase-treated erythrocytes; T, trypsin-treated erythrocytes; C, chymotrypsin-treated erythrocytes.
We further investigated the binding specificity of region 2 of EBL-1 by expressing it as a fusion protein in the pRE4 vector, as described in ref. 15. As a control, we expressed the extracellular region of EBL-1 near the transmembrane sequence. For all transfected constructs, positive surface expression was confirmed by immunofluorescence with three antibodies: two directed against the epitopes on the N terminus and C terminus of the heterologous fusion protein in the vector pRE4 and the third directed against region 2 of EBL-1. Both EBL-1 and the two regions of the pRE4 vector were expressed transiently on the surface of CHO-K1 cells. CHO-K1 cells expressing region 2 of EBL-1 bound to glycophorin B positive (+) erythrocytes, but not to glycophorin B-null erythrocytes of two unrelated donors, indicating that its erythrocyte receptor is glycophorin B (Fig. 5B). The binding of CHO-K1 cells expressing region 2 of EBL-1 to human erythrocytes was sensitive to neuraminidase and chymotrypsin treatment of the erythrocytes, but it was resistant to trypsin (Fig. 5B). The same pattern of rosetting was observed when region 2 of EBL-1 was expressed on COS-7 cells. We found that the rosettes formed by region 2 of EBL-1 contained fewer erythrocytes than the rosettes formed by region 2 of EBA-175 (Fig. 5A). These rosettes were also less dense than those formed by region 2 of BAEBL/EBE1-140 and region 2 of JESEBL/EBE1-181. The region near the transmembrane sequence of EBL-1 did not bind human erythrocytes as would be expected. Region 2 of DBL-EBP in the P. falciparum genes is duplicated (F1 and F2 domains) compared with the P. vivax region 2 where only one copy is observed. The F1 and F2 domains of EBL-1 expressed alone on CHO-K1 cells did not bind to human erythrocytes, indicating that both domains are required for erythrocyte recognition.

Discussion

Here, we have provided evidence that the receptor for EBL-1, a member of the DBL-EBP family of receptors, is glycophorin B. First, EBL-1 from P. falciparum culture supernatant binds to glycophorin B+ and not to glycophorin B-null erythrocytes as indicated by immunoprecipitation with antibodies specific for region 2 of EBL-1. The immunoprecipitated protein was in the range of the predicted size of EBL-1. The antibodies specific for EBL-1 did not precipitate a protein from the supernatant of P. falciparum clone HB3 in which the ebl-1 gene is deleted in the genome (9). Second, region 2 of EBL-1 expressed on the surface of CHO-K1 cells bound glycophorin B+ erythrocytes but not glycophorin B-null erythrocytes in two genetically unrelated glycophorin B-null individuals.

Glycophorin B has long been suspected to be a receptor for P. falciparum because of the high level of polymorphism in individuals living in malaria-endemic regions. However, because P. falciparum has multiple redundant pathways of invasion, no difference has been observed between the invasion of glycophorin B-null and glycophorin B-positive erythrocytes. For example, invasion of erythrocytes by the P. falciparum Dd2/Nm clone that expresses EBL-1 and the HB3 clone that lacks EBL-1 is similar compared with invasion of glycophorin B-positive erythrocytes (Dd2, 79%; HB3, 72%) (16).

EBL-1 and the other members of the DBL-EBP family share a high degree of sequence homology in region 2 and contain three exons at the 3′ end that encode the transmembrane region and the cytoplasmic domain. EBL-1, however, differs in the 3′ cysteine-rich region, the most conserved region of the DBL-EBP family, even between species, suggesting that EBL-1 has a unique function (1). The EBL-1 genes from ~50% of P. falciparum clones have 5 thymidines inserted into the ORF that leads to a frameshift, suggesting that the gene is evolving to be a pseudogene.

Glycophorin B shows a high degree of polymorphism in malaria-endemic areas, including S-s-U-, Henshaw with variable sequence of the first 5 aa, and Miltenberger mutations that have recombination between glycophorin A and B (17). The Miltenberger mutations in malaria-endemic areas always lead to loss of glycophorin B. The most extreme example is the 59% gene frequency on S-s-U- erythrocytes in the Efe pygmies of the Ituri
Forest in the Democratic Republic of Congo (13). This region also has chimpanzees and gorillas that are infected with *Plasmodium reichenowi*, a *P. falciparum*-like *Plasmodium*. Evidence indicates that *P. falciparum* was spread through the world ~100,000 years ago (18), with a more recent expansion of infection with the introduction of agriculture in Africa and the diversification of the *Anopheles gambiae* mosquito complex ~10,000 years ago (19). We can only speculate as to when *P. falciparum* spread to the human population. It may have originated from *P. reichenowi* of the higher apes. It is possible that the pygmies lost glycoporphin B to resist *P. falciparum* after it first entered humans, and then the parasite may have undergone diversification of its invasion pathways and mutations to silence EBL-1. We speculate that the *P. reichenowi* genome may have a smaller number of DBL-EBP family members, one being syntenic with EBL-1.

Theoretical studies published as *SI Text* indicate that a null allele of glycoporphin B would need to afford only a modest level of protection against malaria in heterozygous to increase in frequency from a single mutant to an allele frequency of 0.59 in the relevant time frame. Assuming a constant population of size 1,000–10,000 individuals, one need invoke a selective advantage of only 1% in homozygous-null genotypes to have a single copy of the null mutant allele increase to a frequency of ~59% across an interval of 100,000 years (5,000 generations). A shorter time entails stronger selection, but even for 10,000 years (500 generations), a selective advantage of only 10% in homozygous-null genotypes is required. Both cases require partial dominance corresponding to ~10–20% as much protection in heterozygous genotypes as in the homozygous-null. *Table S1* also includes models of heterozygote superiority, where again the levels of selection that need be invoked are quite modest.

**Materials and Methods**

**Antisera.** Antisera specific for EBL-1 region 2 of Dd2/Nm were generated by immunization of rabbits with a DNA vaccine created with the vector VR1050 kindly supplied by S. Hoffman, Naval Medical Research Center, Silver Spring, MD. This vector contains the T cell epitopes P2P30 from tetanus toxoid. Region 2 gene fragments of EBL-1 were amplified from *P. falciparum* clone Dd2/Nm and cloned into the VR1050 vector, as described by VR1012fPap2p230 in refs. 19 and 20 but now renamed VR1050 (W. O. Rogers, personal communication). The inserts for regions 2 of Dd2/Nm spanned from amino acid D200 to EB42 (GenBank accession no. FJ392548). Rabbits were immunized intradermally with 500 μg of DNA at 3-week intervals for a total of four immunizations. A shorter time entails stronger selection, but even for 10,000 years (500 generations), a selective advantage of only 10% in homozygous-null genotypes is required. Both cases require partial dominance corresponding to ~10–20% as much protection in heterozygous genotypes as in the homozygous-null.

**Immunoblot Analysis.** Equal concentrations of ring, trophozoite, and schizont stage protein samples from the clones Dd2/Nm were extracted in Laemmli sample buffer and separated on 8% SDS/polyacrylamide gels. The proteins were transferred to PVDF membranes. The membranes were blocked in 0% nonfat dry milk in PBS and 1% Tween 20 overnight at 4 °C. The membranes were then incubated with rabbit antibodies directed against DBL-1 and DBL-2 for 1 h at room temperature. After washes, the membranes were incubated with a 1:10,000 dilution of goat anti-rabbit, horseradish peroxidase-conjugated antibodies. The membranes were washed, and antibody complexes were detected with enhanced chemiluminescence (GE Healthcare).

**Erythrocytes Used in the Studies.** Blood was collected in 10% (vol/vol) citrate phosphate dextrose and stored for up to 4 weeks at 4 °C. At the time of study, the erythrocytes were washed three times in incomplete medium (RPMI medium 1640; Life Technologies) with 25 mM Hepes and 0.36 mM hypoxanthine (Sigma).

**Metabolic Labeling of Parasite Proteins.** Soluble, metabolically labeled parasite proteins were obtained from culture supernatant of schizont-infected erythrocytes that released merozoites in the absence of uninfected erythrocytes. The parasites were left to lyse and release proteins into the culture supernatant. The Dd2/Nm and HB3 clones of *P. falciparum* were cultured as described in ref. 7 with the following exceptions. Schizont-infected erythrocytes (5 × 10^9^ per mL of culture medium) were used during the metabolic labeling with 200 μCi/ml [35S]Met/Cys-Met. The culture supernatant was ultracentrifuged in a Beckman Optima TLX ultracentrifuge (Beckman–Coulter) at 40,000 rpm (88,600 × g) for 10 min at 4 °C before storage of the supernatant at 80 °C.

**Immunoprecipitation.** Proteins in the supernatant and in the diluted eluate were immunoprecipitated as described in ref. 7 with the following exceptions. The supernatant (50 μL) was diluted into 250 μL of NETT (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) supplemented with 0.5% BSA (ICN) to decrease background. Radiolabeled supernatant (50 μL) was preabsorbed with protein A-Sepharose as described elsewhere (7). The supernatant was split into 2 equal volumes and immunoprecipitated with 5 μL of anti-EBL-1 region 2 and 1 μL of anti-EBA-175 region 2, as described above. Protein G-Sepharose [40 μL, 50% (vol/vol); GE Healthcare] was added to remove the immune complexes.

**Modified Erythrocyte-Binding Assay.** Erythrocyte-binding assays were performed as described in ref. 7. We found that one or two absorptions with 50 μL of packed erythrocytes to 50 μL of metabolically labeled supernatant were required to remove EBL-1 from the supernatant, depending on the concentration of EBL-1 in the supernatant. Elution from erythrocytes of bound parasite proteins was performed as described in ref. 7. Parasite proteins were eluted only from the erythrocytes of the first adsorption. The parasite proteins were eluted as described above. The eluate was diluted 5-fold (vol/vol) in NETT with 0.5% BSA before immunoprecipitation.

**Immunolocalization of EBL-1.** The methods for immunolocalization of EBL-1 by confocal microscopy were performed as described in ref. 7. The secondary antisera consisted of Alexa Fluor 488-conjugated goat anti-rat IgG, Alexa Fluor 488-conjugated goat anti-mouse IgG, Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) diluted 1:200 in blocking buffer. For antiquenching, we mounted labeled parasites in ProLong Antifade (Invitrogen).

**Cell Culture and Transfection of CHO-K1.** CHO-K1 cells (American Type Culture Collection) were cultured in RPMI medium 1640 as described in ref. 12. The transfected cells were used for immunofluorescence or erythrocyte-binding assays 48 h after transfection.

**Expression of Region 2 of EBL-1 on CHO-K1 Cells and COS-7 Cells.** Region 2 of EBL-1 were cloned into the pRc4 vector, which contains the gene encoding herpes simplex virus glycoprotein D under the control of the Rous sarcoma virus LTR promoter in a mammalian expression vector that has been described (11, 15). The forward primer 5'-GATCAGCTGGTGGATATATCCATAAAG-3' and reverse primer 5'-CCGGGGCTATAGGAAACACATC-3' were used to generate region 2 from Dd2/Nm by using the PvuII and ApaI sites, respectively, from amino acid D200 to EB42 (GenBank accession no. FJ392548). PCR conditions were 94 °C for 2 min, and 30 cycles at 94 °C for 50 s, 55 °C for 30 s, and 72 °C for 3 min.

**Enzyme Treatment of Erythrocytes.** Erythrocytes were collected in 10% citrate phosphate dextrose and stored at 4 °C. Erythrocytes were washed three times in RPMI medium 1640 (pH 7.4) containing 0.5% hypoxanthine (Invitrogen) before use. Erythrocytes were treated with enzymes as described in ref. 12.

**Genetically Deficient Human Erythrocytes.** Fresh glycoporphin B-null erythrocytes (S-S-U) were obtained from two separate donors from the Virginia Blood Services (Richmond, VA) and South Community Blood Center (Gainesville, FL).

**Erythrocyte-Binding Assay on CHO-K1 Cells.** The erythrocyte-binding assay was performed as described in ref. 12. Efficiency was assayed by an immunofluorescence assay as described below. Binding of untreated and enzyme-treated erythrocytes to untransfected CHO-K1 cells and to cells expressing CIDR1 in pRc4 (21), a PEPF1 domain for endothelial cytoadherence, was tested as a negative control. For the immunofluorescence assay, transfected cells were washed in PBS and fixed in 3.7% formaldehyde for 5 min at room temperature. This procedure was followed by three washes in PBS for 1 min each at room temperature. The cells expressing the fusion protein in pRc4 were stained with mouse monoclonal antibodies...
DL6 and ID3 directed against the herpes simplex glycoprotein D sequences as described (11, 15, and gifts from R. Eisenberg and G. Cohen, University of Pennsylvania, Philadelphia), and rabbit polyclonal antisera directed against region II of EBL-1, respectively.

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