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Coxsackieviruses B1, B3, and B5 Use Decay Accelerating Factor as a Receptor for Cell Attachment

DARREN R. SHAFREN,[1]* RICHARD C. BATES,[2] MICHAEL V. AGREZ,[3] ROBERT L. HERD,[1] GORDON F. BURNS,[2] AND RICHARD D. BARRY[1]

Department of Microbiology,[1] Cancer Research Unit,[2] and Department of Surgical Science,[3] Faculty of Medicine, The University of Newcastle, New South Wales 2300, Australia

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Receptor binding and subsequent cell-mediated internalization or disassembly are the initial steps in virus replication. Cell surface molecules that participate in this process are the primary determinants of virus tissue tropism. Monoclonal antibody blockade, immunoprecipitation, and DNA transfection were used to identify decay accelerating factor as a major cell attachment receptor for coxsackieviruses B1, B3, and B5. However, expression of human decay acceleration factor on the surface of nonpermissive murine fibroblasts led only to virus attachment without subsequent replication, and it was concluded that an additional cellular cofactor(s) is required to facilitate cell entry and subsequent replication.

The coxsackie B group viruses are small, nonenveloped, single-stranded viruses belonging to the genus *Enterovirus* in the family *Picornaviridae*. Infection of newborns and infants by these viruses can induce paresis, aseptic meningitis, and febrile illnesses that can be fatal, while adult infections are more commonly asymptomatic (9). Coxsackie B group viruses are considered the main causal agents of virus-induced myocardial disease (15, 29, 33), infect a wide range of cells in the immune system, and are thought to be involved in virus-induced autoimmune diseases (32, 34).

Early evidence as to the nature of the cellular receptor for a hemagglutinating strain of coxsackie B3 virus (CB3) evolved through work on the capacity of picornaviruses to hemagglutinate human type O erythrocytes (RBCs) (22). Echoviruses 7 (E7), 11, and 19 and coxsackievirus B3 (hemagglutination-positive strain) were shown to attach to the same molecule on the surface of human type O RBCs, and detergent preparations of the membranes of these cells inhibited the replication of these viruses in permissive HeLa cells (23). Virus attachment interference assays indicated that CB1 and CB3 share a trypsin-resistant receptor on the surface of HeLa cells which is distinct from that used by polioviruses (5, 36). The HeLa cell attachment receptor for group B coxsackieviruses was considered to be a multicomponent glycoprotein complex possessing an approximate molecular mass of 275 kDa (14). A monoclonal antibody (MAb) raised against a 49.5-kDa membrane protein isolated from sucrose gradient-purified CB3-HeLa cell receptor complexes inhibited the replication of all group B coxsackieviruses but not that of polioviruses and E6 (12, 18). However, a MAb generated against intact HeLa cells inhibited the replication of only CB1, CB3, CB5, E6, and coxsackievirus A21 in HeLa cells; this MAb recognized a 60- to 70-kDa cell surface protein detected by immunoblotting (7). These two membrane proteins are considered distinct saturable receptors on the surface of HeLa cells and constitute part of the larger multicomponent receptor complex used by B group coxsackieviruses (7, 12, 14).

* Corresponding author. Mailing address: Discipline of Pathology, Faculty of Medicine, Level 3, David Maddison Clinical Sciences Building, Royal Newcastle Hospital, Newcastle, New South Wales 2300, Australia. Phone: 61 49 23 6158. Fax: 61 49 23 6148. Electronic mail address: MDDRS@Newcastle.cc.edu.au.

In this paper, data based on both MAb blockade and DNA transfection of nonpermissive murine fibroblasts indicate that a cell surface-binding receptor for CB1, CB3, and CB5 is decay accelerating factor (DAF). However, DAF alone is insufficient to facilitate cell entry and lytic infection of permissive cells.

Inhibition of the binding and replication of CB1, CB3, and CB5 in HEp-2 cells by MAb 854. The initial aim of this study was to identify the cellular receptor(s) involved in E7 infection by using MAb 854, selected for its ability to inhibit the replication of E7 (Wallace) in susceptible cells (24). As E7 and CB3 share cell surface receptors on type O RBCs, we also investigated the ability of MAb 854 to inhibit cell infection by coxsackieviruses B1 to B6. Plaque formation by 50 to 70 PFU of CB1 (Conn-5), CB3 (Nancy), and CB5 (Faulkner) but not by CB2 (Ohio), CB4 (Benschoten), and CB6 (Schmitt) or poliovirus type 3 (PV3 Sabin) was selectively inhibited in HEp-2 monolayers in six-well tissue culture plates that had been pretreated with 50 μg of MAb 854 per ml (Fig. 1). A control MAb, 280, directed against the poliovirus receptor (PVR) (20) inhibited only PV3-induced plaque formation (Fig. 1). Furthermore, in microtiter plate infectivity assays, MAb 854 blocked E7 infection approximately 100- to 1,000-fold more than it did CB1, CB3, or CB5 infection (data not shown). Next, we investigated whether MAb 854 inhibited the lytic infection of HEp-2 cells by CB1, CB3, and CB5 by blocking viral attachment or by interfering with the cell entry mechanism. To address this, HEp-2 cell monolayers were pretreated with MAb 854 or 280 (50 μg/ml) and then incubated with approximately 2×10^5 cpm of ^{35}S-labeled CB1 to CB6 or PV3. The viruses E7, CB1 to CB6, and PV3 were labeled with [^{35}S]methionine and purified on sucrose velocity gradients (18), and their purity was confirmed by polyacrylamide gel electrophoresis (PAGE) and autoradiography (data not shown). MAb 854 selectively inhibited the attachment of E7 (data not shown), CB1, CB3, and CB5 by between 65 and 90% without significantly affecting the binding of PV3, CB2, CB4, and CB6; these findings are comparable to those of Crowell et al. (7). MAb 280 selectively inhibited only PV3 binding (Fig. 2).

MAb 854 recognizes DAF. Since the E7 receptor has recently been reported as DAF (3), it seemed likely that MAb 854 is directed at a DAF epitope, and as a consequence, DAF may also be the attachment receptor for CB1, CB3, and CB5. DAF is a 70-kDa glycosyl-phosphatidylinositol-anchored protein

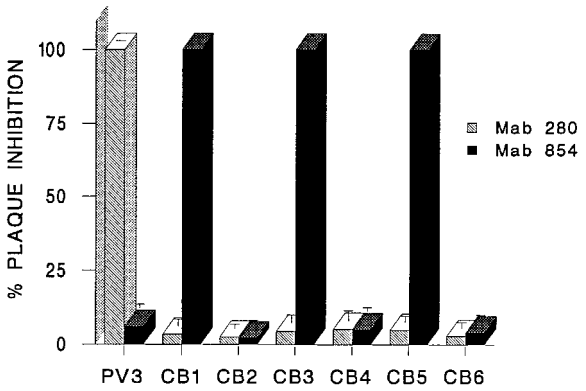

FIG. 1. Inhibition of CB1, CB3, and CB5 plaque formation by MAb 854. Confluent monolayers of HEp-2 cells in six-well tissue culture plates were incubated for 30 min at 37°C with serum-free Dulbecco minimal essential medium containing MAb 854 or 280 (50 μg/ml). Virus (~50 to 70 PFU/well) was added and incubated for 1 h at 37°C before the cells were overlaid with 5.0 ml of agar overlay. Following incubation at 37°C for 72 h, plaques were visualized by staining with crystal violet solution. Results are expressed as mean percent inhibitions relative to the no-MAb control for triplicate wells plus two standard deviations.

that protects cells from complement-mediated lysis by preventing either the formation or the association of C3 convertases and consists of four short consensus repeats (SCR) and a serine-tyrosine-enriched region providing potential O-linked glycosylation sites (16, 17, 21). To examine this possibility, the nature of the MAb 854 binding molecule was determined as follows. WOP cells (polyomavirus-transformed mouse 3T3 fibroblasts that do not bind CB1, CB3, or CB5), susceptible HeLa cells, HEp-2 cells, and human type O RBCs were incubated with MAb 854, and binding was analyzed by flow cytometry. The fluorescence histograms shown in Fig. 3A indicate that, as expected, MAb 854 bound to the surfaces of HeLa and HEp-2 cells and RBCs but not to the surfaces of the WOP cells. Consequently, HEp-2 cells and RBCs were surface labeled by lactoperoxidase-catalyzed iodination essentially as described previously (2) prior to lysis in an octylglucoside lysis buffer (100 mM octylglucoside, 150 mM sodium chloride, 2

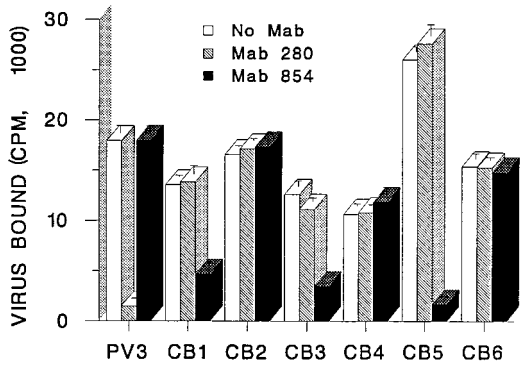

FIG. 2. Inhibition of radiolabeled CB1, CB3, and CB5 attachment to HEp-2 cells by MAb 854. HEp-2 monolayers in 24-well tissue culture plates were first incubated for 1 h with MAbs 280 and 854 (50 μg/ml) in serum-free Dulbecco minimal essential medium at 37°C and then were washed and incubated with the labeled virus (2×10^5 cpm) in serum-free medium for the same period. Following three washes, the cell monolayers were dissolved in 200 μl of 0.2 M NaOH–1.0% SDS and the amount of labeled virus that was bound was measured with a scintillation counter. Results are expressed as the means of triplicate wells plus two standard deviations.

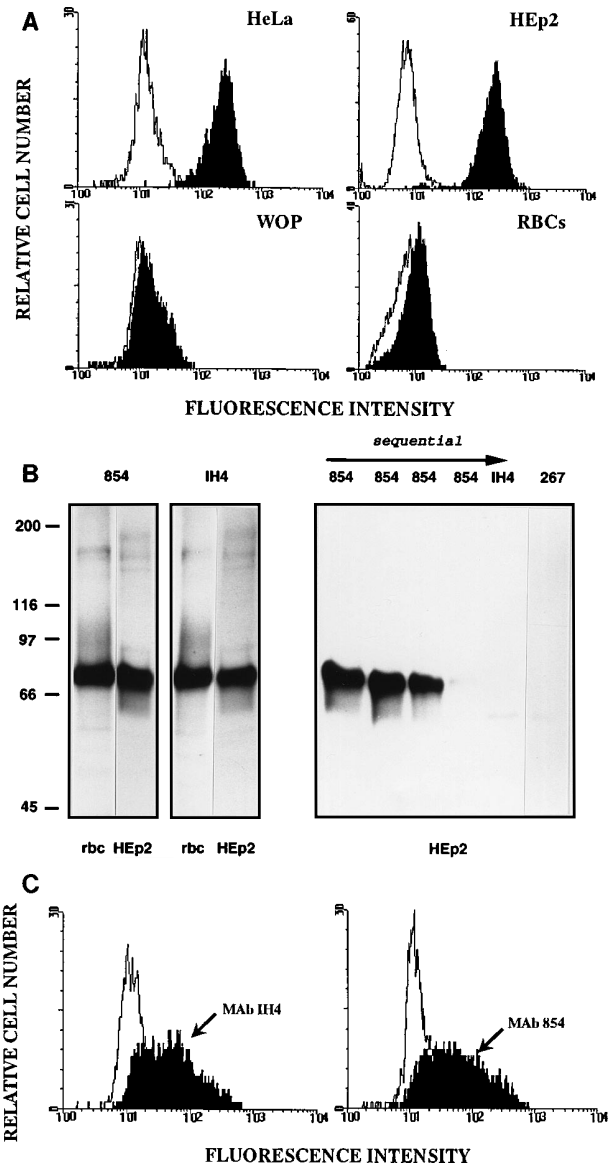

FIG. 3. Characterization of MAb 854. (A) Binding of MAb 854 to the surface of susceptible and nonsusceptible cells and RBCs. Cells (10^6) in 100-μl aliquots were incubated with MAb 854 or an isotype-matched MAb diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) (PBS-BSA) on ice for 30 min, after which the cells were washed with 5.0 ml of PBS-BSA. The cells were then pelleted at $1,000 \times g$ for 5 min and resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (heavy plus light chains) (Silenus, Melbourne, Australia) diluted in PBS-BSA. Following incubation on ice for 30 min, the cells were washed and pelleted as described before, resuspended in 100 μl of PBS-BSA, and analyzed with a FACStar analyzer (Becton Dickinson, Sydney, Australia). The open graphs represent binding of the isotype-matched control MAb; the solid graphs represent binding of MAb 854. (B) Immunoprecipitation of DAF from radioiodinated HEp-2 cells and RBCs. Lysates of the two cell types labeled on the cell surface were immunoprecipitated with MAb 854 and the anti-DAF MAb IH4 as described in the text. Analysis of the immunoprecipitates by SDS-PAGE and autoradiography identified similar 70-kDa bands precipitated by both MAbs from each cell type. The identity of the band for HEp-2 cells is shown in the right panel, which shows that sequential depletion of the lysate of the 854 antigen totally removed any DAF precipitated by MAb IH4. Immunoprecipitation of the nondepleted lysate with control MAb 267 (a poliovirus type 2 neutralizing MAb) is shown as a measure of nonspecific binding. The gels were exposed to autoradiography for 16 h. The numbers at the left are kilodaltons. (C) Binding of MAb 854 to DAF-transfected WOP cells. WOP cells transiently expressing human DAF were incubated with MAbs IH4 and 854 and an isotype-matched MAb control and subjected to analysis by flow cytometry as described in the legend for Fig. 3A. The open graphs represent binding of the isotype-matched MAb; the solid graphs represent binding of MAbs IH4 and 854.

mM phenylmethylsulfonyl fluoride, 20 mM iodoacetamide, and 50 μg of soybean trypsin inhibitor per ml) for 1 h. Cell lysates were centrifuged at 10,000 × *g* for 10 min. For immunoprecipitations, the lysates were precleared with rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark) coupled to Sepharose 4B beads (Pharmacia, Uppsala, Sweden) for a minimum of 2 h. Immunoprecipitations were carried out indirectly by incubation with primary antibodies followed by precipitation with rabbit anti-mouse immunoglobulins–Sepharose 4B and analyzed by sodium dodecyl sulfate (SDS)–7.5% PAGE and autoradiography. Immunoprecipitation of HEp-2 cells and RBCs with MAb 854 identified a protein of approximately 70 kDa from both preparations (Fig. 3B), which is approximately the molecular mass of DAF (16, 17, 21). Further immunoprecipitation analysis of these membrane preparations with an anti-DAF MAb (IH4) identified a single polypeptide with a size identical to that of the protein recognized by MAb 854. Sequential immunoprecipitation of ^{125}I-labeled HEp-2 cells with MAb 854 directly coupled to Sepharose 4B beads depleted all the precipitable by itself, because no further material could be precipitated subsequently by IH4 (Fig. 3B). MAb IH4 was tested by indirect immunoprecipitation (Fig. 3B). To further confirm the immunoprecipitation findings, WOP cells transiently expressing human DAF were reacted with MAbs 854 and IH4 and the relative bindings of the MAbs were assessed by flow cytometry. As shown in Fig. 3C, cell surface-expressed human DAF was detected by both MAbs 854 and IH4 with similar sensitivities. These findings indicate that MAb 854 recognizes an epitope on the DAF molecule.

CB1, CB3, and CB5 can bind to DAF. To determine whether human DAF possessed the capacity to function as a cellular attachment receptor for CB1, CB3, and CB5, WOP cells in the exponential phase of growth were trypsinized, washed, and resuspended in electroporation buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], pH 7.05; 137 mM NaCl; 5 mM KCl; 0.7 mM Na_2HPO_4, 6 mM glucose) at a concentration of 5×10^6 to 5×10^7 cells/ml. Aliquots (500 μl) of the cells were mixed with 50 μg of PVR-pAmR8 or DAF-pAmR8 in electroporation cuvettes (Bio-Rad, Richmond, Calif.) and pulsed at 300 V and 250 μF with a Bio-Rad gene pulser. The expression vector pAmR8 is an analog of pCDM8 (30), differing only in the replacement of the SupF selection system with an ampicillin resistance gene. Following a 10-min recovery period at room temperature, the cells were resuspended in Dulbecco minimal essential medium containing 10% fetal calf serum, seeded in 24-well tissue culture plates, and incubated for 48 h at 37°C in 5.0% CO_2. Flow cytometric analysis with MAbs 854 and 280 revealed that approximately 35% of the cell populations expressed detectable levels of DAF or PVR. These cells were then incubated with approximately 10^5 cpm of ^{35}S-labeled preparations of CB1, CB3, CB5, E7, and PV3. (E7 and PV3 served as positive controls.) The results (Fig. 4A) showed that E7, CB1, CB3, and CB5 bound to a significantly greater extent to DAF-transfected cells than to PVR-expressing cells. Labeled PV3 bound only to cells expressing the PVR (Fig. 4A). To confirm the fidelity of viral attachment and to begin to map the virus binding domain, DAF-expressing WOP cells were preincubated with MAbs 854, IH4 (anti-DAF SCR 3 [4]), and IA10 (anti-DAF SCR 1 [13]) or with the anti-PVR MAb 280 and then incubated with ^{35}S-labeled E7 or CB5. It was found that MAb 280 had no effect on reducing E7 or CB5 binding. Of the anti-DAF MAbs, IA10 partially inhibited the binding of both E7 and CB5 while MAbs 854 and IH4 reduced viral attachment to near background levels (Fig. 4B). These data indicate that CB1, CB3, and CB5 utilize DAF as a primary binding receptor on the surface of susceptible cells and that E7 and CB5 bind to adjacent regions within or proximal to the SCR 3 domain of DAF.

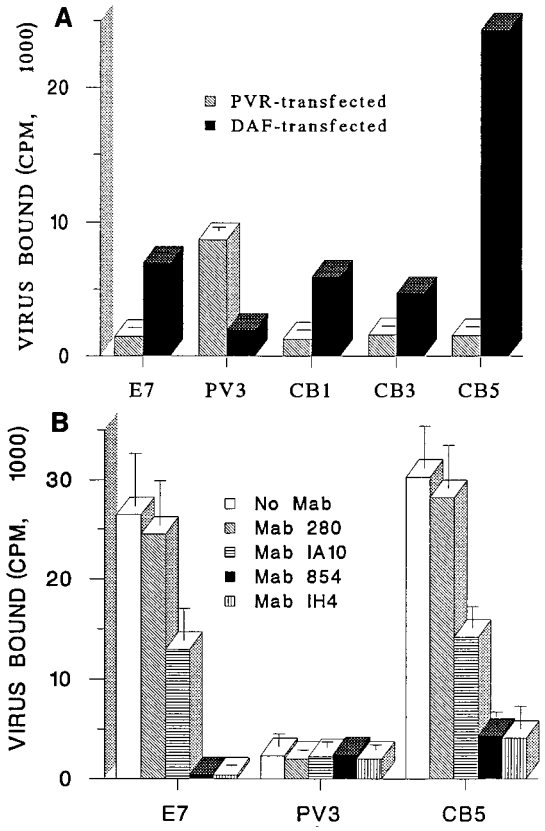

FIG. 4. Binding of CB1, CB3, and CB5 to DAF-expressing WOP cells. (A) WOP cells transfected with the cDNA for either human DAF or PVR were incubated in 24-well plates with 2×10^5 cpm of ^{35}S-labeled CB1, CB3, CB5, E7, and PV3 as described in the legend to Fig. 2. (B) Inhibition of CB5 and E7 binding by anti-DAF MAbs. WOP-transfected cell monolayers were preincubated with MAbs 280, IA10, IH4, and 854 (50 μg/ml each) and incubated with 2×10^5 cpm of ^{35}S-labeled E7 and CB5 as described in the legend to Fig. 2. Results are expressed as the means of triplicate wells plus two standard deviations.

To determine whether DAF-expressing WOP cells could support replication of CB1, CB3, and CB5, DAF- and PVR-transfected WOP cells were inoculated at a multiplicity of infection of 10 PFU/cell and the cell monolayers and maintenance medium were harvested at 0, 24, and 48 h postinoculation and assayed for infectious virus by plaque assay on HEp-2 cell monolayers. WOP cells expressing DAF did not support replication of CB1, CB3, or CB5, whereas the PVR-expressing cells allowed replication of PV3 at levels of up to 10^2 PFU/ml at 48 h postinoculation (data not shown). However, WOP cells transfected with CB3 RNA produced infectious CB3 virions (data not shown), implying that the failure of the viruses discussed above to infect DAF-expressing WOP cells may have been due to a cell entry block.

We have thus established that the DAF molecule is used as a attachment receptor in the lytic infection cycle of CB1, CB3, and CB5. Cell attachment and replication of CB1, CB3, and CB5 were inhibited by the pretreatment of susceptible cells with MAb 854, an antibody that is shown to recognize an epitope on the DAF molecule. Antibody blockade with anti-DAF MAbs identified the possible binding domain for CB5 and E7 as a region on or proximal to the third SCR of the DAF molecule. These data are consistent with previous findings that

the binding domain of E7 resides on either SCR 2 or SCR 3 of DAF (3). The ability of MAb 854 to inhibit the lytic infection of E7 100- to 1,000-fold more efficiently than that of CB1, CB3, and CB5 suggests that the coxsackieviruses may attach to primary receptors other than DAF. Another explanation is that the CB1, CB3, and CB5 prototype preparations contained a mixed population of hemagglutination-positive and -negative virions with various affinities for DAF while the E7 population consisted of a more homogeneous population of virions with a high affinity for DAF. These data are consistent with the hypothesis that CB3 utilizes a multicomponent cellular attachment site (7, 12, 14, 18). In this regard, it is of interest to note that CB1, CB3, and CB5, in addition to binding to DAF, are distinguishable from CB2, CB4, and CB6 by their higher affinities for agglutination of human type O RBCs (11) and the ability to attach to susceptible cells at significantly faster rates (6).

The third SCR of DAF is believed to be crucial for intracellular signalling, and only antibodies directed against this epitope inhibit DAF function (4, 17, 21). Removal of SCR 1 has no effect on DAF function, but deletion of SCR 2, SCR 3, or SCR 4 significantly impairs DAF function (4). DAF is known to mediate signal transduction through the *src* family of kinases, i.e., $p56^{lck}$ and $p59^{fyn}$ (8, 31). Recent findings have indicated that DAF and many other glycosyl-phosphatidylinositol-anchored proteins are concentrated in cellular structures known as caveolae, structures proposed as potential sites for transmembrane signal transduction (28, 35). Additionally, caveolae are known to transcytose macromolecules (e.g., modified low-density lipoprotein and albumin) via a pathway independent of coated-pit endocytosis (10, 27).

We show here that CB5 binds selectively to a domain on or near the third DAF SCR and not to the N-terminal SCR, as might be predicted by the canyon hypothesis (26). Even though CB1, CB3, and CB5 possess capsid architectures similar to those of other picornaviruses, e.g., polioviruses and human rhinovirus 14, they appear to attach to cell membranes by a different mechanism. Polioviruses and human rhinovirus 14 attach to susceptible cells via a specific interaction of the N-terminal immunoglobulin-like domain of PVR (19) and intercellular molecule 1 (26), respectively, with viral epitopes located at the floor of a canyon positioned at the pentameric apex of viral capsid protein 1. One explanation for this difference may be that CB5 attachment to the third SCR initiates a specific signal transduction pathway that may activate either accessory cellular factors or other intracellular molecules that can participate in the virus internalization and/or other stages in the viral replication cycle.

Considering that DAF is highly expressed on most mammalian cells (1, 17, 21, 25, 37), we postulate that the cell and tissue tropism of CB1, CB3, and CB5 is governed by the presence or absence of specific accessory and internalization cofactors or the inability of certain cell types to construct the correct signal transduction pathway(s) to facilitate its activation. This is exemplified by the inability of DAF-transfected murine fibroblasts (this report) and DAF-expressing rabbit kidney cells (30a) to support multicycle replication and lytic infection by these viruses. From the results reported here, it might be anticipated that such cell membrane cofactors may be identified within or adjacent to membrane caveolae.

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