The β Subunit of Human Granulocyte-Macrophage Colony-stimulating Factor Receptor Forms a Homodimer and Is Activated via Association with the α Subunit

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Summary

Human granulocyte-macrophage colony-stimulating factor (hGM-CSF) receptor (hGMR) consists of α and β subunits, and the precise stoichiometry of these subunits has remained to be determined. In this work, oligomerization of the β subunit was studied using a chemical cross-linker. In Ba/F3, a mouse interleukin-3–dependent cell line expressing both subunits of hGMR (Ba/F3-α,β), a protein with a molecular mass corresponding to that of a homodimer of the β subunit (β homodimer) was detected only when cells were treated with the cross-linker. Dimerization of the β subunit was confirmed by coimmunoprecipitation of a tagged β subunit with the wild type β subunit in COS7 cells. The β homodimer had already formed in the absence of hGM-CSF, whereas stimulation with the ligand brought both α and β subunits into a complex, the result being tyrosine phosphorylation of the β homodimer. Tyrosine phosphorylation of the β subunit was impaired by deletion of the cytoplasmic domain of the α subunit without interfering with the association of both subunits. These results indicate that the β homodimer, which alone is insufficient for signaling, forms the functional hGMR with the α subunit in response to hGM-CSF.

G M-CSF is a secreted protein regulating proliferation and differentiation of hematopoietic cells (1). The high-affinity and functional GM-CSF receptor (GMR) consists of α and β subunits, and both subunits are single transmembrane proteins belonging to the cytokine receptor superfamily (1). The α subunit is specific to GMR, and the β subunit is shared by high-affinity receptors for IL-3 and IL-5.

The high-affinity GMR is reconstituted when both subunits are coexpressed in various species of cells (2, 3). Analyses using deletion mutants of the α and β subunits revealed that the cytoplasmic domains of both subunits are required for signal transduction but not for binding with GM-CSF (4, 5). Mutation analyses for GM-CSF also indicated that the ligand forms a heterotrimeric complex with both α and β subunits (6). On the basis of these findings, the functional GMR was thought to be a heterodimer composed of α and β subunits. However, the possibility that the functional GMR exists as a larger complex containing multiple α and β subunits rather than a heterodimer had to be excluded.

Recent studies revealed that functional receptors take on configurations of subunits varying from receptor to receptor (7). Functional forms of many cytokine receptors as well as GMR are composed of two distinct subunits. Some of these receptors, such as the IL-2R, require heterodimerization for activation of the subunits (8). The IL-6R also consists of two types of subunits, the IL-6R α subunit and gp130, its functional form is a heterotetramer composed of two molecules each of two subunits but not a heterodimer (9), and homodimerization of gp130 was shown to be involved in activation of the receptor (10). Thus, the stoichiometry of the subunits has to be determined to better understand the mechanism of activation of GMR.

We report here evidence that the β subunit of the human GMR (hGMR) formed a homodimer (β homodimer), irrespective of human GM-CSF (hGM-CSF) stimulation. The β homodimer, which was tyrosine phosphorylated after association with the α subunit in response to hGM-CSF, is likely to become a component of the functional hGMR. The cytoplasmic domain of the α subunit was required for tyrosine phosphorylation of the β subunit.

Materials and Methods

Chemicals, Media, and Antibodies. Recombinant hGM-CSF produced in *Escherichia coli* was provided by the Schering-Plough

Corp. (Kenilworth, New Jersey). Mouse IL-3 (mIL-3) produced by silkworms (*Bombyx mori*) was purified as described previously (11). RPMI 1640 and DME were purchased from Nikken Bio-Medical Laboratories Co., Ltd. (Kyoto, Japan). FCS was purchased from Gibco Life Technologies, Inc. (Gaithersburg, MD). Bis(sulfosuccinimidyl)suberate (BS^3) was from Pierce Chemical Co. (Rockford, IL). Horseradish peroxidase–linked sheep anti–mouse Ig and the enhanced chemiluminescence (ECL) system were purchased from Amersham Japan Co. Ltd. (Tokyo, Japan). Mouse mAb against hGMR α subunit GMA-1 and rat mAb against human common β subunit 5A5 (12) were prepared in our laboratory and were used for immunoprecipitation. Rabbit polyclonal antibodies against the extracellular domain (anti–β[e]) and the cytoplasmic domain (positions of amino acid residues 453–544; anti–β[c]) of the human common β subunit, respectively, were obtained from Medical & Biological Laboratory Co. Ltd. (Nagoya, Japan). Antiphosphotyrosine mouse mAb 4G10 was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Murine ascites specific to the hemagglutinin (HA) epitope (anti-HA) was obtained from Berkeley Antibody Co. (Richmond, CA).

Cell Lines and Culture Conditions. An mIL-3–dependent pro–B cell line Ba/F3 was maintained in RPMI 1640 medium containing 10% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 0.25 ng/ml mIL-3. An hGM-CSF–dependent preerythroid cell line, M-TAT (13), was obtained from Dr. M. Yamamoto (Tsukuba University, Tsukuba, Japan). The cells were maintained in the same medium as Ba/F3, except for 1 ng/ml hGM-CSF instead of mIL-3. A monkey kidney cell line, COS7, was maintained in DME containing 10% FCS, 50 U/ml penicillin, and 50 μg/ml streptomycin.

Construction of Mutant Receptors. The truncated mutants of the α and β subunits, α_{328} and β_{455}, were constructed as described elsewhere (4). To construct the β-HA subunit cDNA, a short DNA fragment encoding the epitope for the anti-HA antibody was linked to the downstream of *Fsp*I site of the truncated β_{455} cDNA. The amino acid sequence encoded by the epitope tag is P^{455}SMYPYDVPDYASLGGSSSS. All cDNAs were joined downstream of the SRα promoter in pME18S.

Preparation of Protein Samples from Hematopoietic Cells. After factor depletion, the cells were washed and resuspended in PBS at 10^7 cells/ml. The cells were stimulated with or without 10 ng/ml hGM-CSF for 10 min at 4°C. The cell surface proteins were cross-linked by treatment with indicated concentrations of BS^3 for 20 min at 4°C, and the reactions were quenched by addition of 100 mM Tris-HCl, pH 7.5. The cells were solubilized in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 50 mM NaF, 100 μM Na$_3$VO$_4$, 1 mM PMSF, 1 μg/ml pepstatin, and 2 μg/ml leupeptin for 1 h at 4°C. After centrifugation, the supernatant was incubated with an indicated antibody for 1 h at 4°C followed by further incubation in the presence of protein G–Sepharose for 1 h at 4°C. The immunoprecipitate from 10^7 (Ba/F3) or 2 × 10^7 (M-TAT) cells was analyzed by Western blotting.

Transfection of COS7 Cells. COS7 cells were transfected by the DEAE–dextran method. Cells were plated at 7 × 10^5 cells/10-cm plate at day 0. On day 1, the growing cells were washed with PBS and incubated for 4 h at 37°C in DME containing DNA and 500 μg/ml of DEAE–dextran without FCS. 2.5 μg each of two plasmid DNAs (receptor cDNAs or the control plasmid) were used for each transfection. At day 3, the transfected cells were harvested and solubilized in lysis buffer containing 0.5% of Triton X-100 instead of NP-40. The β and β-HA subunits were immunoprecipitated and analyzed by Western blotting.

Results

Chemical Cross-Linking of Two Molecules of the β Subunit. We first examined molecule(s) associated with the β subunit using a chemical cross-linker in combination with Western blotting. The chemical cross-linker BS^3 is a hydrophilic reagent and can react with cell surface proteins but not with cytoplasmic proteins (14). Ba/F3-α,β cells (15), which express both α and β subunits of hGMR, were stimulated with hGM-CSF and then treated with various concentrations of BS^3. The β subunit was then immunoprecipitated with an anti–β subunit antibody (5A5) and analyzed by Western blotting using the anti-β(e) antibody (both antibodies are hereafter referred to as "anti-β"). In
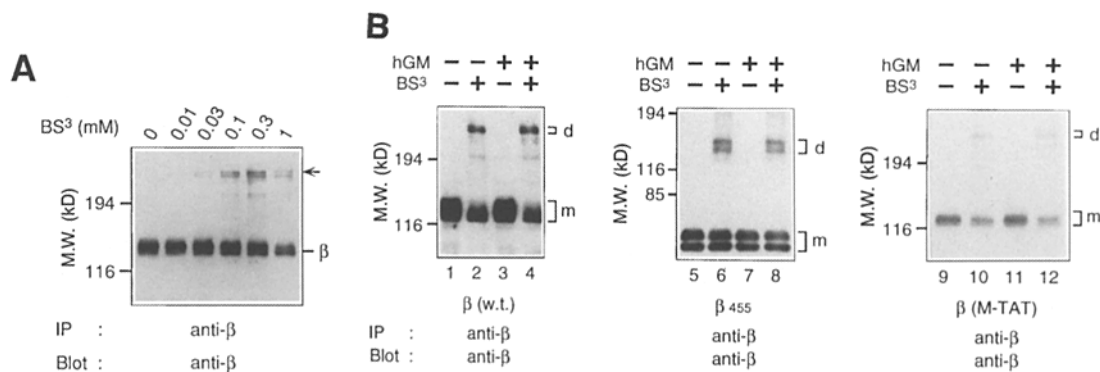

Figure 1. The β subunit forms a homodimer even in the absence of hGM-CSF in Ba/F3 cells. (*A*) Titration of BS^3. After stimulation of Ba/F3-α,β cells with hGM-CSF, the cells were incubated with the indicated concentrations of BS^3 as described in Materials and Methods. Immunoprecipitates with 5A5 were analyzed by Western blotting using the anti-β(e) antibody. Both 5A5 and the anti-β(e) antibody are represented as "anti-β." The positions of the monomeric β subunit (β) and the cross-linked complex (*arrow*) are shown on the right. (*B*) Ba/F3-α,β cells (lanes *1–4*), Ba/F3-α,β$_{455}$ cells (lanes *5–8*), and M-TAT cells (lanes *9–12*) were stimulated with (lanes *3, 4, 7, 8, 11*, and *12*) or without (lanes *1, 2, 5, 6, 9*, and *10*) hGM-CSF, and their surface proteins were cross-linked with (lanes *2, 4, 6, 8, 10*, and *12*) or without (lanes *1, 3, 5, 7, 9*, and *11*) 0.3 mM BS^3. The cross-linked proteins were immunoprecipitated and analyzed by Western blotting under the same conditions described in *A*. The positions of the monomeric (*m*) and dimeric (*d*) β or β$_{455}$ subunits are shown on the right.
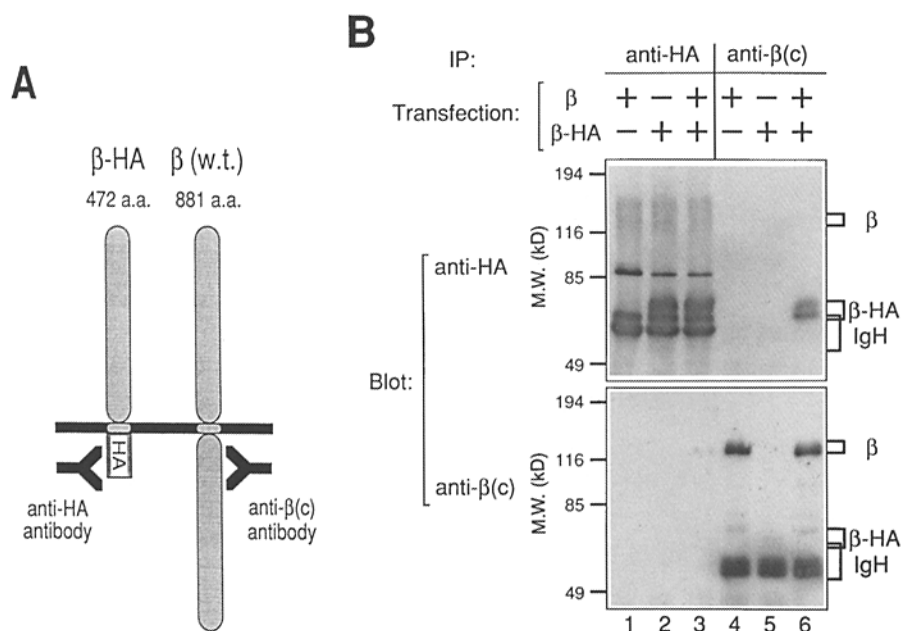
B

A

Figure 2. Formation of the β-homodimer in COS7 cells. (A) Schematic representation of the structures of the wild-type β [β(w.t.)] and chimeric β-HA subunits and modes of recognition by the anti-β(c) and anti-HA antibodies. (B) COS7 cells were transfected with each of the β(w.t.) (lanes 1 and 4) and β-HA (lanes 2 and 5) subunit cDNAs, or with both (lanes 3 and 6), by the DEAE–dextran method. Proteins were immunoprecipitated using either the anti-HA (lanes 1–3) or anti-β(c) (lanes 4–6) antibody, and then analyzed by Western blotting using the (A) anti-HA and (B) anti-β(c) antibodies. Positions of the wild type β and β-HA subunits and an IgH chain are shown on the right.

the absence of BS³, only a monomeric β subunit (β monomer) was detected as a 130-kD protein (Fig. 1 A). Treatment with tunicamycin, an inhibitor of glycosylation, revealed that the duplicated bands of the β monomer were due to differences in glycosylation (data not shown). Upon treatment with BS³, there was evidence of an additional 260 kD protein recognized by the anti-β antibody. The molecular mass of this protein was twice as large as that of the β monomer. The most efficient cross-linking was observed at 0.3 mM BS³, and a higher concentration of BS³ slightly impaired the efficiency of immunoprecipitation with the anti-β antibody. The β monomer was always detected under these conditions. Thus, only part of the β subunit can be cross-linked. Whether only a fraction of the β subunit exists in the 260-kD complex or whether the cross-linking reaction used was inefficient was not determined.

To determine if the 260-kD BS³-linked protein was composed of two molecules of the β subunit, we did cross-linking experiments using Ba/F3-α,β$_{455}$ cells, which express the wild type α subunit and the mutant subunit β$_{455}$ lacking most of the cytoplasmic domain (4). The β$_{455}$ monomer was detected as a 75-kD protein, and an additional 150-kD protein was seen, depending on chemical cross-linking, with 0.3 mM BS³ (Fig. 1 B, lanes 7 and 8). These results strongly suggest that two molecules of the β or β$_{455}$ subunit were cross-linked with BS³, under the conditions of our experiment.

The cross-linked 260- or 150-kD protein was found even in the absence of hGM-CSF (Fig. 1 B, lanes 2 and 6). The addition of hGM-CSF did not affect patterns of the bands. The chemically cross-linked 260-kD protein, which is similar to that in Ba/F3-α,β cells, was also observed in an hGM-CSF–dependent preerythroid cell line, M-TAT (13) (Fig. 1 B, lanes 10 and 12). Western blotting after SDS-PAGE under nonreducing conditions revealed that forma-

tion of the protein complex was not mediated by a disulfide bond (data not shown).

Coimmunoprecipitation of Two Molecules of the β Subunits. We also confirmed that the β subunit forms a homodimer, determined by coimmunoprecipitation analyses using a transient expression system in COS7 cells. To distinguish between two β subunits coimmunoprecipitated, we constructed the β subunit with an epitope tag (β-HA; Fig. 2 A). The β-HA subunit is a chimeric β subunit in which all of the cytoplasmic domain is replaced by the HA epitope tag, and the deduced molecular mass is 78 kD. COS7 cells were transfected with either the wild-type β or β-HA subunit cDNA or both. After solubilization of the cells, immunoprecipitation followed by Western blotting were done using either an antibody against the cytoplasmic domain of the β subunit (anti-β[c]) or an anti-HA antibody. The wild-type β and chimeric β-HA subunits were specifically recognized by the anti-β(c) and anti-HA antibodies, respectively (Fig. 2, A and B, lanes 1, 2, 4, and 5). When both subunits were coexpressed in COS7 cells, they coimmunoprecipitated by either the anti-HA or anti-β(c) antibody, in the absence of hGM-CSF (Fig. 2 B, lanes 3 and 6). Since the β-HA subunit lacks the cytoplasmic domain of the β subunit, interaction between two β subunits is likely to be mediated by extracellular and/or transmembrane domains. This is consistent with the result of the chemical cross-linking experiment using Ba/F3-α,β$_{455}$ cells (Fig. 1 B). Taken together, the β subunit was shown to form a non–disulfide-linked homodimer through the extracellular and/or transmembrane domains, regardless of hGM-CSF stimulation.

The β Homodimer Can Be a Component of a Functional hGMR. To characterize the β homodimer as a component of a functional hGMR, we examined the association with the α subunit and tyrosine phosphorylation of itself. Ba/F3-α,β cells were stimulated with hGM-CSF and
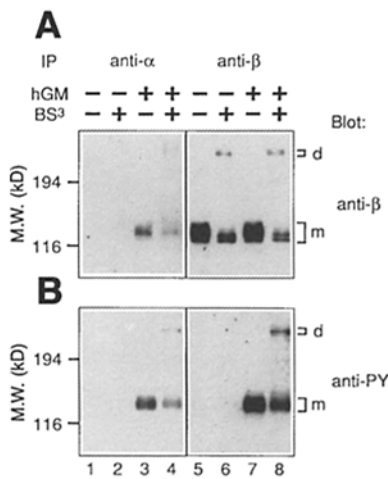
A

B

Figure 3. The β-homodimer is a component of a functional hGMR. Ba/F3-α,β cells were stimulated with (lanes *3, 4, 7,* and *8*) or without (lanes *1, 2, 5* and *6*) hGM-CSF, and cell surface proteins were then cross-linked with (lanes *2, 4, 6,* and *8*) or without (lanes *1, 3, 5,* and *7*) 0.3 mM BS³. Proteins were immunoprecipitated using either GMA-1 (lanes *1–4*) or 5A5 (lanes *5–8*) and then analyzed by Western blotting using (*A*) anti-β(e) and (*B*) antiphosphotyrosine (anti-PY) (4G10) antibodies. Positions of the monomeric (*m*) and dimeric (*d*) β subunits are shown on the right.

treated with BS³, and lysates were then used for immuno-precipitation as described for Fig. 1 *B*. Both the monomer and the BS³–cross-linked dimer of the β subunit were immunoprecipitated by an anti–α subunit antibody in a manner dependent on stimulation with hGM-CSF (Fig. 3 *A*, lanes *3* and *4*). Accordingly, the cross-linked β homodimer formed a stable complex with the α subunit only in the presence of hGM-CSF. Since stimulation with hGM-CSF preceded cross-linking with BS³, it is likely that the β subunit actually existed as a dimer in the complex with the α subunit, in the absence of the cross-linker.
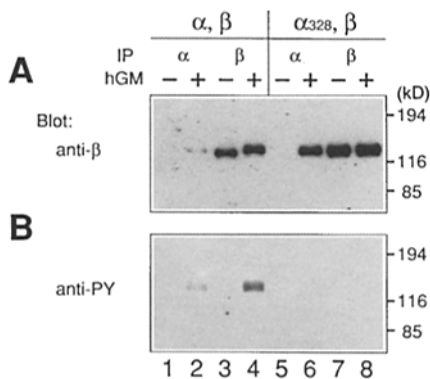
A

B

Figure 4. The β subunit was activated via interaction with the cytoplasmic domain of the α subunit. Ba/F3-α,β (lanes *1–4*) and Ba/F3-α₃₂₈,β (lanes *5–8*) cells were used. The cells were stimulated with (lanes *2, 4, 6,* and *8*) or without (lanes *1, 3, 5,* and *7*) hGM-CSF. Proteins were immunoprecipitated using either GMA-1 (lanes *1, 2, 5,* and *6*) or 5A5 (lanes *3, 4, 7,* and *8*) antibody and analyzed by Western blotting using (*A*) the anti-β(e) antibody and (*B*) 4G10.

As the β homodimer as well as the β monomer were tyrosine phosphorylated in response to hGM-CSF (Fig. 3 *B*, lanes *3, 4, 7,* and *8*), homodimerization of the β subunit is apparently not sufficient for activation of hGMR in the absence of hGM-CSF. Consistent with this notion, the addition of the cross-linker did not affect growth of Ba/F3-α,β cells in the presence or the absence of hGM-CSF (data not shown). Although it seems that the β homodimer can be part of the functional hGMR and appears to be in an inactive state, without hGM-CSF, the β monomer may also be a functional component. Further investigations are needed to show that the β homodimer is indeed essential for signaling through hGMR.

Activation of the β Subunit Requires Interaction with the Cytoplasmic Domain of the α Subunit. The preformed (or inactive) β homodimer was activated via an hGM-CSF–dependent association with the α subunit. We then examined the requirement of the cytoplasmic domain of the α subunit for the activation of hGMR, using the mutant α subunit α_{328} which lacks most of the cytoplasmic domain. The α_{328} subunit did associate with the β subunit in response to hGM-CSF (Fig. 4 *A*, lane *6*). However, tyrosine phosphorylation of the β subunit was not induced in Ba/F3-α₃₂₈,β cells (Fig. 4 *B*, lane *6*), a finding consistent with previous observations that the cytoplasmic domain of the α subunit is required for signal transduction but not for reconstitution of a high-affinity hGMR (4, 5). Therefore, interaction of the cytoplasmic domains of the α subunit with the β homodimer is an important step needed to activate hGMR. It remains to be determined whether the activation of hGMR is due to a conformational change of the β subunit or to recruitment of a molecule(s) associated with the cytoplasmic domain of the α subunit.

Discussion

We obtained evidence that the β subunit of hGMR forms a homodimer even in the absence of hGM-CSF and that the β homodimer appears to be a component of a functional hGMR. The precise role of dimerization of the β subunit in triggering hGMR signaling is unclear. Oligomerization of receptors has been noted for various receptors for cytokines and growth factors (16). Oligomeric forms of the receptors are required for activation of intrinsic tyrosine kinases and for transphosphorylation of growth factor receptors. Although members of the cytokine receptor superfamily have no known activity for intrinsic tyrosine kinase, they do associate with cytoplasmic tyrosine kinases such as Janus kinases (JAK) (17); the β subunit but not the α subunit of hGMR was shown to associate with JAK2 (18). Mechanisms involved in activation of cytokine receptors may be similar to those functioning for growth factor receptors, and JAK kinases may possibly be activated through association with oligomerized receptors, which in turn would oligomerize the kinases (17). If so, then the β-homodimer may be required for oligomerization (or activation) of JAK2 or other related kinases.

We also found that homodimerization of the β subunit is not sufficient for activation of hGMR, and further interaction with the cytoplasmic domain of the α subunit is required. Although the mechanism to activate the β subunit is unclear, a proline-rich motif conserved in the cytoplasmic domains of the α subunits of GMR, IL-3R, and IL-5R seems to be important for signaling through the common β subunit (5, 19) (Muto, A., unpublished results). In addition, our recent study showed that the cytoplasmic domain of the α subunit can be replaced by that of the β subunit when reconstituting the functional hGMR (15). The cytoplasmic domain of the β subunit also contains a proline-rich motif termed box1. Therefore, the three α subunits, by using the proline-rich motif, may function similarly with regard to the mechanism of activation of the β subunit.

Recently, Jenkins et al. (20) reported that the β subunit of hGMR was constitutively activated by point mutations. One (V449E) of these mutations was found in the transmembrane domain, and this resembled the activating mutation (V664E) found in the *neu* oncogene, which had been shown to activate the Neu protein by inducing constitutive dimerization (21). On the basis of the similarity, they spec-

ulated that the V449E mutant of the β subunit might be activated by ligand-independent dimerization. However, we found that the wild-type β subunit forms a homodimer, even in the absence of the ligand. Thus, the mutant β subunit is likely to be activated by mechanisms other than the homodimerization mediated by the mutation.

The precise stoichiometry of the α and β subunits remains an open question since we detected no oligomeric form of the α subunit. Nevertheless, several lines of evidence suggest that α and β subunits exist in a molar ratio of one to one in a functional hGMR. Most cytokines, including hGM-CSF, are composed of four α helices and have similar tertiary structures (22). Mutation analyses of hGM-CSF revealed that its first and fourth helices were binding sites for the β and α subunits, respectively (6). In addition, a crystallographic study revealed that one molecule of growth hormone with a tertiary structure similar to that of hGM-CSF associates with two molecules of monomeric receptors through the first and fourth helices (23). It is therefore tempting to speculate that two molecules of hGM-CSF bring two α subunits and one β homodimer into a high-affinity hGMR complex.

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