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Antiserum to carcinoembryonic antigen recognizes a phosphotyrosine-containing protein in human colon cancer cell lines

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Members of the carcinoembryonic antigen (CEA) family include CEA, non-specific cross reacting antigen (NCA), and biliary glycoprotein (BGP), and appear to function as cell adhesion molecules. Immunoprecipitation and subsequent gel electrophoresis of proteins from several colon cancer cell lines labeled with [γ-^{32}P]ATP, under conditions designed to detect ecto-kinase-catalyzed phosphorylation of cellular proteins, revealed that polyclonal anti-CEA antiserum recognized a 175–190 kDa phosphoprotein on the surface of colon cancer cells. The ability to detect this phosphoprotein did not correlate with CEA production, and immunoprecipitation studies suggested that the phosphoprotein is BGP. Phosphoamino acid analysis of the 175–190 kDa protein showed that it contained predominantly phosphotyrosine.

Biliary glycoprotein; Carcinoembryonic antigen; Ectoprotein kinase; Protein phosphorylation; Phosphotyrosine

1. INTRODUCTION

Carcinoembryonic antigen (CEA) was first described as a 180 kDa tumor-associated cell surface glycoprotein on colon cancer cells [1,2]. Subsequently, a number of closely related, cross-reacting antigens have been found in normal cells [3–6]. The CEA gene belongs to a family of at least 20 closely related genes which belong to the immunoglobulin gene superfamily [3–6]. The CEA gene family consists of two major subgroups. The CEA subgroup contains genes that encode CEA, non-specific cross reacting antigen (NCA), and biliary glycoprotein (BGP), while the other subgroup consists of genes that encode the pregnancy-specific glycoproteins [6]. The general structure of members of the CEA family consists of a 34 amino acid hydrophobic leader sequence, an N-terminal domain of 107–110 amino acids that is similar to the immunoglobulin variable domain, and a variable number of immunoglobulin C2-like domains, in addition to a short hydrophobic carboxy-terminal region that is post-translationally modified to allow membrane anchoring via a glycosyl-phosphatidylinositol linkage [5,7]. The different members of the CEA family can be distinguished by the number of repeated C2 subunits; CEA consists of 6 repeats, NCA has 2 repeats, and BGP has 3 repeats. Until recently, all CEA and NCA molecules were thought to be linked to the plasma membrane via a glycosyl-phosphatidylinositol linkage [8–11]. In contrast, BGP has been shown to have a transmembrane domain and a cytoplasmic domain [11,12]. Members of the CEA family appear to function as cell–cell adhesion molecules [13–15].

Protein phosphorylation is an important mechanism of regulation of cell function. Although most studies of protein phosphorylation have been directed at intracellular reactions, evidence has been provided for the existence of ecto-protein kinase activity on the surface of several different types of cells (reviewed in [16–18]). This ecto-protein kinase is capable of utilizing extracellular ATP to phosphorylate exogenous substrates as well as endogenous surface proteins. The role of ecto-protein kinase activities in cell function is unknown. One of the major endogenous substrates for human neutrophil ecto-protein kinase activity was phosphorylated predominantly on tyrosine residues and was immunoprecipitated by both CD15 and CD66 monoclonal antibodies [19,20]. This protein is also recognized by polyclonal anti-CEA and thus may represent BGP [20]. In addition, CD66 antibodies were found to react with CEA, NCA, and BGP [20]. We thus questioned whether CEA or related proteins might be phosphorylated in colon cancer cells. This report demonstrates that polyclonal anti-CEA recognizes a ~175–190 kDa phosphoprotein in several human colon cancer cell lines. Phosphorylation of this protein was detectable when cells were radi-

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Abbreviations: CEA, carcinoembryonic antigen; NCA, non-specific cross reacting antigen; BGP, biliary glycoprotein; FBS, fetal bovine serum; PMSF, phenylmethylsulfonylfluoride; NRS, normal rabbit serum; PAGE, polyacrylamide gel electrophoresis; P-S, phosphoserine; P-T, phosphothreonine; P-Y, phosphotyrosine.

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olabeled in the presence of [γ-^{32}P]ATP utilizing a technique designed to detect ecto-protein kinase activity, but was not detected when cells were metabolically radiolabeled with $H_3{}^{32}PO_4$. Phosphoamino acid analysis of this protein revealed that it contained predominantly phosphotyrosine with a lower level of phosphothreonine/phosphoserine.

2. MATERIALS AND METHODS

2.1. *Cell lines*

The human colon cancer cell lines COLO 201, COLO 205 [21], HCT 116 [22], and LoVo [23], were obtained from the American Type Culture Collection, Rockville, MD. The colon cancer cell lines KM12 and KM12SM [24], melanoma cell lines A375 and A375SM [25], and renal cell carcinoma cell lines SN12 and SN12PM-6 [26] were gifts of Dr. I. Fidler, MD Anderson Cancer Center, Houston, TX.

2.2. *^{32}P-Labeling*

Cells were radiolabeled with ^{32}P by using two different techniques. In the first method, 2.5×10^7 cells were incubated for 2 h at 37°C in 750 μl of a buffer containing 145 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, and 5 mCi of $^{32}PO_4$ (carrier-free orthophosphate, Amersham). DFP was then added to 10 mM and the cells incubated for 5 min at 23°C. The cells were then collected by centrifugation at $9,800 \times g$ for 2 min and solubilized and analyzed as described below.

In the second labeling procedure [19,20], 2×10^7 cells were incubated for 15 min at 23°C in 1 ml of a buffer containing 1 mM $MnCl_2$, 145 mM NaCl, 20 mM HEPES, pH 7.4, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, and 0.5 mCi [γ-^{32}P]ATP (specific activity 4,500 Ci/mmol, ICN, Irvine, CA). These conditions were previously found to result in efficient protein radiolabeling by ecto kinase activity [19,20]. At the end of the incubation, DFP was added to 10 mM, the cells were incubated 23°C for 5 min, and recovered by centrifugation at $9,800 \times g$ for 2 min. The cells were then solubilized and analyzed as described below.

2.3. *Immunoprecipitation and PAGE*

Immunoprecipitation was performed as previously described [20]. Briefly, 2×10^7 radiolabeled cells were suspended in 1.2 ml of cell solubilization buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5% NP-40, 1 mM Na_3VO_4, 1 mM Na_2MoO_4, 100 μM ATP, 0.02% NaN_3, and 1 mM phenylmethylsulfonylfluoride (PMSF)) and incubated on ice for 15 min. The suspensions were then centrifuged at $9,800 \times g$ for 15 min at 4°C, and the supernatant used for immunoprecipitation or analyzed by SDS-PAGE directly. Rabbit anti-CEA was obtained from Accurate Chemical Corp., Westbury, NY. Monoclonal antibody T84.1, which recognizes CEA and BGP, and monoclonal antibody T84.6, which recognizes CEA but not BGP, were gifts of Drs. R. Paxton and S. Hefta, City of Hope, Duarte, CA.

2.4. *Phosphoamino acid analyses*

Phosphoamino acid analyses were performed as previously described [27]. Briefly, radiolabeled polypeptides resolved by SDS-PAGE were localized by autoradiography, excised, and eluted from gel slices. After precipitation with 20% TCA, the polypeptides were subjected to acid hydrolysis in vacuo in 6 M HCl at 100°C for 3–4 h, and the resultant phosphoamino acids were resolved by one-dimensional thin layer chromatography on aluminum sheets precoated with silica gel (0.2 mm layer thickness, 11 cm height) (Merck Laboratories, EM Science, Gibbstown, NJ) in ethanol: 25% NH_4OH (3.5:1.6). The chromatography cycle was repeated three times to achieve optimal separation. Between cycles, plates were air dried followed by chromatography in the same solvent.

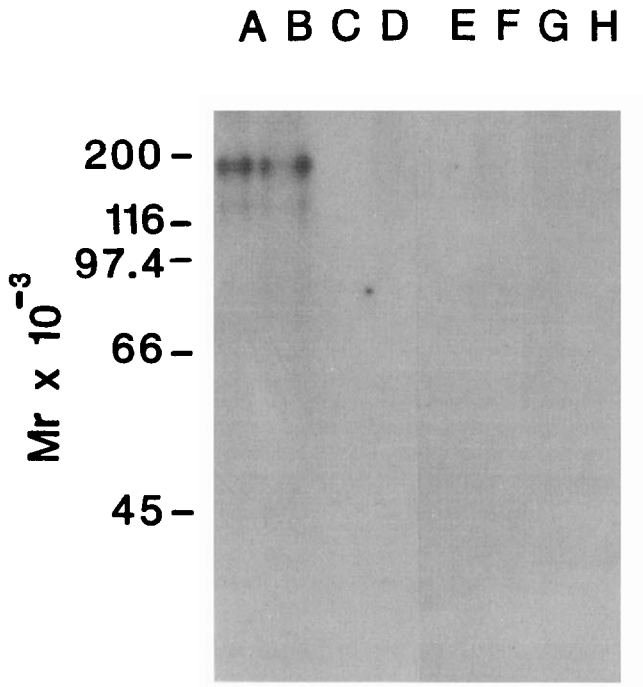

Fig. 1. Immunoprecipitation and SDS-PAGE of colon cancer, melanoma, and renal cell cancer cells radiolabeled with [γ-^{32}P]ATP. KM12 (lanes A and C), KM12SM (lanes B and D), A375 (lane E), A375SM (lane F), SN12 (lane G), and SN12PM-6 (lane H) cells were radiolabeled with ^{32}P by the addition of [γ-^{32}P]ATP, solubilized, and immunoprecipitated with rabbit anti-CEA (lanes A,B and E–H) or NRS (lanes C,D), and analyzed by SDS-PAGE and autoradiography as described in the text. Proteins used as molecular weight standards were: myosin heavy chain, 200,000; *E. coli* β-galactosidase, 116,000; phosphorylase a, 97,400; BSA, 66,000; and ovalbumin, 45,000.

3. RESULTS

3.1. *Immunoprecipitation of ^{32}P-labeled proteins by anti-CEA*

To determine if proteins recognized by anti-CEA in colon cancer cell lines were phosphorylated, two approaches to radiolabeling were taken. First, viable KM12, KM12SM, COLO 201, and COLO 205 cells were incubated with $H_3{}^{32}PO_4$ to metabolically radiolabeled phosphoproteins. However, anti-CEA did not immunoprecipitate any detectable ^{32}P-labeled proteins from these cell lysates (not shown). In the second approach, based on previous studies with neutrophils [20], viable colon cancer cells were incubated with [γ-^{32}P]ATP, a technique designed to detect phosphoproteins whose phosphorylation is catalyzed by intrinsic ecto-protein kinase activity. This labeling technique resulted in ^{32}P incorporation into several proteins, with most ^{32}P incorporation found in proteins of ~120–250 kDa (not shown). Immunoprecipitation of extracts of KM12 cells radiolabeled in this manner with anti-CEA resulted in the identification of a ^{32}P-labeled protein of ~175–190 kDa (Fig. 1, lane A). A ~125–145 kDa ^{32}P-labeled protein of lower intensity was also seen. Normal rabbit serum (NRS) did not immunoprecipitate these

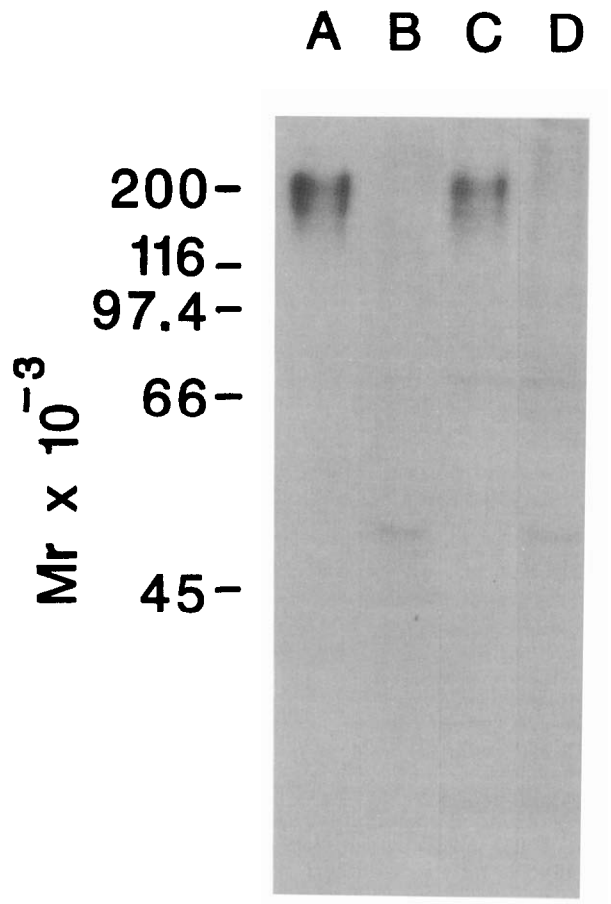

Fig. 2. Immunoprecipitation and SDS-PAGE of ^{32}P-labeled proteins from colon cancer cells radiolabeled with [γ-^{32}P]ATP. Colo 201 (lanes A and B) and Colo 205 (lanes C and D) cells were radiolabeled with ^{32}P by the addition of [γ-^{32}P]ATP, solubilized, and immunoprecipitated with rabbit anti-CEA (lanes A and C) or NRS (lanes B and D), and analyzed as in Fig. 1.

proteins (lane C): Anti-CEA also immunoprecipitated ^{32}P-labeled proteins of similar molecular weight from extracts of KM12SM cells radiolabeled by this technique (lane B) while NRS did not (lane D).

Four other cell lines, A375, A375SM (melanoma), SN12, and SN12PM-6 (renal cell carcinoma), were also radiolabeled by this technique; however, anti-CEA did not immunoprecipitate any ^{32}P-labeled proteins from extracts of A375 (Fig. 1, lane E), A375SM (lane F), SN12 (lane G), or SN12PM-6 (lane H) cells. To further characterize the phosphorylation of CEA-related molecules, four other colon cancer cell lines with various levels of CEA production were analyzed. HCT 116 produces CEA at a low level (~1 ng/10^6 cells/ml/10 days) [28] while LoVo produces CEA at a high level (~900 ng/10^6 cells/ml/10 days) [28]. However, anti-CEA did not immunoprecipitate any detectable ^{32}P-labeled proteins from extracts of either LoVo or HCT 116 cells (not shown). In contrast, anti-CEA immunoprecipitated two ^{32}P-labeled proteins of ~175–190 kDa and 125–145 kDa from both COLO 201 (Fig. 2, lane A) and COLO 205 (lane C) while NRS (lanes B and D) did not. COLO 205 produces CEA at a low level (~2 ng/10^6 cells/ml/10 days) [28] while COLO 201 does not produce CEA [28]. However, COLO 201 and COLO 205 produce similar amounts of BGP [13]. T84.1 immunoprecipitated the same ^{32}P-labeled proteins from KM12 cells as did polyclonal anti-CEA (not shown). This monoclonal antibody recognizes CEA and BGP [29]. In contrast, T84.6 recognizes CEA, but not BGP [29], and did not immunoprecipitate these proteins (not shown). We conclude that the ^{32}P-labeled 175–190 kDa protein is BGP.

3.2. *Characterization of the phosphorylation of the phosphoproteins recognized by anti-CEA*

To determine the identity of the ^{32}P-labeled amino

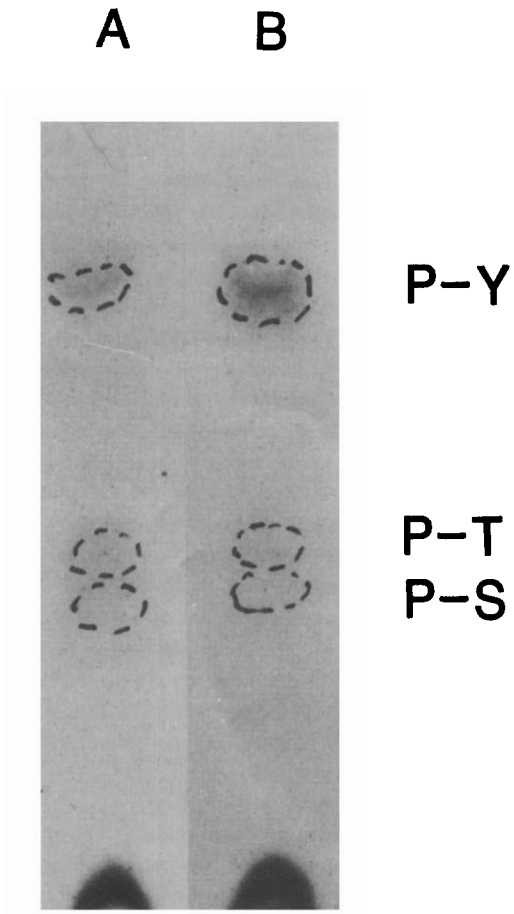

Fig. 3. Phosphoamino acid analysis of phosphoprotein recognized by anti-CEA from KM12 (lane A) and KM12SM (lane B) cells. Cells were ^{32}P-labeled by the addition of [γ-^{32}P]ATP, solubilized, and immunoprecipitated by anti-CEA. The 175–190 kDa phosphoprotein immunoprecipitated by anti-CEA was resolved by SDS-PAGE as in Fig. 1, excised, eluted from the gel slice, and subjected to acid hydrolysis. The resultant phosphoamino acids were resolved by thin layer chromatography and visualized by autoradiography as described in the text. The positions of migration of authentic phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) are indicated by dotted lines.

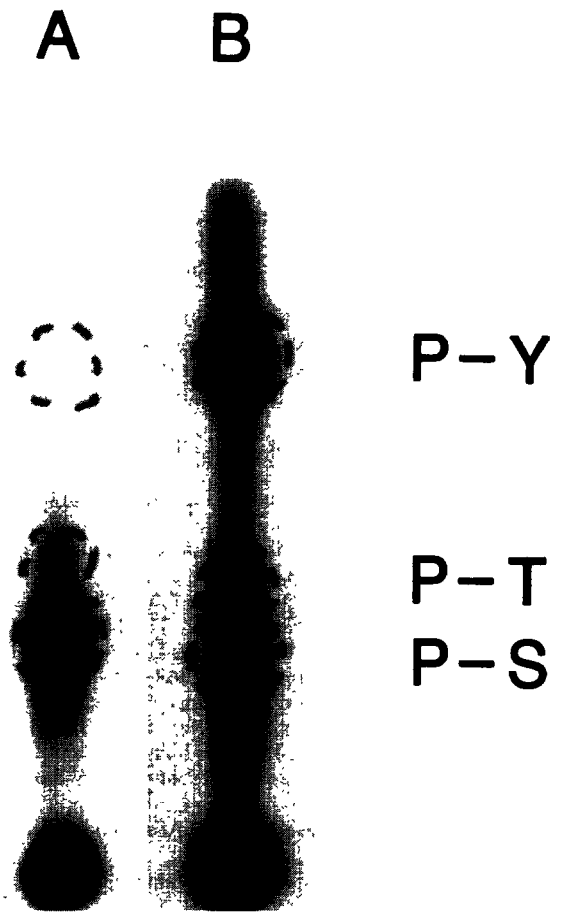

Fig. 4. Phosphoamino acid analysis of whole cell extracts of KM12 cells labeled metabolically by incubation with $H_3{}^{32}PO_4$ (lane A) or by the addition of [γ-^{32}P]ATP (lane B). Cells were radiolabeled by incubation with $H_3{}^{32}PO_4$ or [γ-^{32}P]ATP as described in the text, solubilized, and the soluble proteins were resolved by SDS-PAGE, eluted from the gel, and subjected to acid hydrolysis. The resultant phosphoamino acids were resolved by thin layer chromatography and visualized by autoradiography as in Fig. 3.

acid residues in the proteins recognized by anti-CEA when colon cancer cells were labeled with [γ-^{32}P]ATP, the protein was analyzed for its phosphoamino acid composition. The majority of ^{32}P identified was in phosphotyrosine residues (Fig. 3) with a lower level of phosphothreonine/phosphoserine. To further explore the nature of the ^{32}P incorporation into cellular proteins observed with the addition of [γ-^{32}P]ATP and $H_3{}^{32}PO_4$, extracts of whole cells labeled by these two techniques were analyzed directly for their total phosphoamino acid content. When KM12 cells were metabolically labeled with $H_3{}^{32}PO_4$, most of the ^{32}P was incorporated into serine residues with little incorporation into tyrosine residues (Fig. 4, lane A). In contrast, when cells were radiolabeled by the addition of [γ-^{32}P]ATP, much of the ^{32}P was found in tyrosine residues in addition to serine and threonine residues (lane B).

4. DISCUSSION

CEA is widely used as a tumor marker, and is one of a family of more than 20 genes including NCA and BGP which form a subset of the immunoglobulin gene superfamily [6]. Recent studies have suggested that CEA, NCA, and BGP function as cell adhesion molecules [13–15]. The demonstration that CD66 antibodies react with CEA, NCA, and BGP, and also recognize a 180 kDa neutrophil membrane protein that is phosphorylated under conditions designed to detect ecto-protein kinase activity [20] led us to look for phosphorylation of CEA in colon cancer cells.

In this study, phosphorylation of the CEA-reactive protein was detected when cells were labeled by the addition of [γ-^{32}P]ATP but not by incubation with $H_3{}^{32}PO_4$. These results are consistent with phosphorylation of an external domain by an ecto-protein kinase, as was hypothesized for CD66 [20], but may represent phosphorylation of a cytoplasmic domain. Clearly, the two ^{32}P-labeling techniques result in very different patterns of protein phosphorylation. In particular, as expected since phosphotyrosine is felt to represent < 1% of the phosphorylated residues in total cellular protein [27], metabolic radiolabeling with $H_3{}^{32}PO_4$ resulted in most of the ^{32}P in the whole cell extract being incorporated into serine residues with little incorporation into tyrosine. In contrast, when cells were radiolabeled by the addition of [γ-^{32}P]ATP, much of the ^{32}P in the whole cell extract was incorporated into tyrosine residues, in addition to serine and threonine. However, definitive identification of the phosphorylation site will require further structural studies.

Phosphoamino acid analysis revealed that most of the ^{32}P identified in the anti-CEA reactive protein was in phosphotyrosine residues with a lower level of phosphothreonine/phosphoserine. Receptors for a number of growth factors are phosphorylated on tyrosine; these receptors have intrinsic tyrosine-kinase activity, and their activities may be regulated by phosphorylation [30–33]. In addition, phosphorylation of other cell adhesion molecules has been suggested to be a mechanism of regulating their adhesion functions [34–38].

It is noteworthy that the ability to immunoprecipitate a ^{32}P-labeled protein with anti-CEA from the colon cancer cell lines tested did not correlate well with the reported CEA production by the cell lines. LoVo cells produce CEA at a very high level [28], but anti-CEA did not immunoprecipitate any detectable ^{32}P-labeled proteins from these cells. However, anti-CEA did immunoprecipitate a ^{32}P-labeled protein from COLO 205 cells, which produce CEA at ~0.2% the rate of production by LoVo cells, and also from COLO 201 cells, which do not produce detectable CEA [28]. These results suggest that the phosphoprotein observed in this study is a member of the CEA family but not CEA itself. This conclusion is supported by the ability to im-

munoprecipitate the phosphoprotein by T84.1, which recognizes CEA and BGP, but not by T84.6, which recognizes CEA but not BGP. Thus, the data suggest that the 175–190 kDa phosphoproteins are forms of BGP.

Recently, Afar et al., reported that BGP_a and BGP_b can be phosphorylated on serine and tyrosine residues in LR-73 cells transfected with cDNAs for these proteins [39]. These proteins were also phosphorylated in KG-1 leukemia cells. Interestingly, they detected phosphorylation of BGP_a and BGP_b when cells were labeled with $H_3{}^{32}PO_4$ but not with [γ-^{32}P]ATP, and concluded that the most likely site of phosphorylation is in the cytoplasmic domain. The reason for these differences may lie in the conditions under which the incubation with [γ-^{32}P]ATP was performed. Regardless, the phosphorylation of the CEA-reactive protein on tyrosine observed here suggests a potential role of phosphorylation in the regulation of its function.

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