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Deficiency of cyclic AMP-dependent protein kinases in human psoriasis

(human psoriatic fibroblasts/psoriatic erythrocytes)

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ABSTRACT **To determine possible differences in the cyclic AMP-dependent protein kinases of normal and psoriatic human fibroblasts, the levels of the regulatory subunits (RI and RII, respectively) of protein kinase I and protein kinase II were quantitated by photoaffinity labeling with 8-azido[^{32}P]cAMP. The level of RII was significantly decreased, or was undetectable, in cytosol prepared from fibroblasts from five psoriatic subjects when compared to RII levels found with normal human fibroblasts. The level of cytosolic RI was decreased in fibroblasts from four psoriatic patients and was within the normal range for one diseased patient when compared to RI levels in normal human fibroblasts. The elution profile from a DEAE-cellulose column of protein kinase activity in the soluble fraction from two psoriatic patients also showed a decrease in type I kinase activity and the complete absence of type II kinase activity. Other results indicate that the level of RI in erythrocyte membranes from psoriatic subjects is significantly decreased when compared to that of erythrocyte membranes from eight normal subjects. A significant correlation ($P < 0.001$) was observed between the severity of the cutaneous manifestation of the disease and the level of RI in psoriatic erythrocyte membranes. The changes noted in the levels of RI and RII in cell types other than those thought to be specifically involved in the proliferative epidermis disorder of the disease suggest a general protein kinase deficiency.**

Psoriasis lesions are an example of benign hyperproliferation and abnormal differentiation (1, 2). Epidermal hyperplasia associated with an inflammatory process is the most prominent indication of the disease (3), but psoriasis is not limited to the skin, since it can also affect joints, blood vessels, and perhaps other organs (3–5).

cAMP has been implicated as a modulator of cellular growth and differentiation in a variety of cell types (6, 7). The cAMP-dependent protein kinases appear to be largely, if not solely, responsible for carrying out the biological effects of cAMP (8, 9). Two forms, referred to as type I and type II, of protein kinase have been identified. These forms differ in the nature of their regulatory subunits (RI and RII, respectively); their catalytic subunits appear to be identical (10). Subcellular localization of the two types of protein kinase varies in different tissues. Recent evidence in several cell systems is consistent with the view that protein kinase I has a positive role in cell proliferation whereas protein kinase II is involved in cell differentiation and growth inhibition (11–13).

Although variable results have been reported concerning the intracellular levels of cAMP present in psoriatic tissues (14, 15), experimental evidence suggests a role for cAMP in the manifestation of this hyperproliferative skin disease (14, 16). Further, an abnormal protein phosphorylation pattern has been noted in erythrocyte membranes obtained from psoriatic subjects (17). Thus, it is of interest to determine whether protein kinase activities might be altered in psoriatic patients. In this communication we describe the characterization of the protein kinase present in psoriatic fibroblasts and erythrocytes and present evidence that the decrease in protein kinase levels correlates well with the severity of the disease.

MATERIALS AND METHODS

Reagents. Trypsin (twice crystallized), collagenase (type II-5), cAMP, and histone 2A were purchased from Sigma. Ham's F12 medium and glutamine were from Flow Laboratories; fetal calf serum was from Seromed (Munich, F.R.G.). [γ-^{32}P]ATP was obtained from Amersham, and 8-azido[^{32}P]-cAMP was from ICN. NaDodSO$_4$/PAGE molecular weight standards (low) were from Bio-Rad.

Fibroblast Culture. Human fibroblasts were isolated from normal and untreated adult psoriatic patients by enzymatic digestion of small pieces (6 mm) of dermis from skin biopsies taken from involved skin of the forearm. The skin fragment was rinsed with two 5-ml portions of warm (37°C) Ham's F12 culture medium containing penicillin (50 units/ml) and streptomycin (50 μg/ml). The washed skin sample then was minced and the small pieces obtained were incubated with trypsin (2.5 mg/ml) in 10 ml of Ham's F12 medium for 1 hr. The trypsinized skin fragments were isolated by low-speed centrifugation (100 rpm for 3 min) and then incubated in Ham's F12 medium containing collagenase (1 mg/ml) for 12 hr at 37°C with shaking in a rotatory incubator (New Brunswick, Edison, NJ). Cells were isolated from this incubation mixture by centrifugation at 400 × *g* for 10 min; the cell pellet was then suspended in 5 ml of Ham's F12 medium containing 20% fetal calf serum, penicillin (50 units/ml), and streptomycin (50 μg/ml); and cells were plated onto a 100-mm cell culture dish. Cells were grown in humidified 5% CO_2/95% air at 37°C and the culture medium was changed every 3 days. For protein kinase determinations the cells were used after their fourth or fifth passage.

Preparation of Erythrocyte Membrane. Erythrocyte membranes were prepared according to a modification of the procedure of Dodge *et al.* (18). Ten milliliters of heparinized blood was obtained and immediately centrifuged for 30 min at 1000 × *g* at 4°C. The erythrocyte pellet was suspended in 10 ml of 0.172 M Tris·HCl (pH 7.6) and washed three times with 10 ml of this buffer. The washed erythrocytes were then lysed by suspension in six volumes of hypotonic 0.011 M

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Abbreviations: RI and RII, regulatory subunits of protein kinase I and protein kinase II.

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Tris·HCl (pH 7.6) and membranes were isolated by centrifugation at 2000 × g for 40 min. The membrane pellet was washed three times with 0.011 M Tris·HCl (pH 7.6) and stored frozen at −80°C until used for assay.

DEAE-Cellulose Chromatography. For DEAE-cellulose fractionation, 8×10^7 cells grown on dishes were disrupted in 5 ml of buffer A (10 mM sodium phosphate, pH 7.6/0.2 mM EDTA) with a Dounce homogenizer (30 strokes). The homogenate was centrifuged at 50,000 × g for 30 min and the supernatant (20 mg of protein) was applied to a DE52-cellulose column (0.6 × 13 cm) previously equilibrated with buffer A. The column was washed with 10 ml of buffer A and then eluted with a linear gradient from 20 ml of buffer A to 20 ml of buffer A/0.3 M NaCl. The flow rate was 0.25 ml/min and fractions of 1 ml were collected. Protein kinase activity was determined in each fraction by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to histone, as described by Corbin *et al.* (19). The reaction was initiated by the addition of 30 μl of the fraction being analyzed to 25 mM Mes, pH 7.0/5.0 mM $Mg(OAc)_2$/0.1 mM [γ-^{32}P]ATP (≈200 cpm/pmol) containing 300 μg of histone in a total volume of 50 μl. After incubation at 37°C for 10 min, 25-μl aliquots of the reaction mixture were spotted onto filter paper strips (Whatman 3MM) and the strips were dropped into cold 5% trichloroacetic acid. A blank value obtained by running the reaction without enzyme was subtracted from total ^{32}P incorporation.

Photoaffinity Labeling with 8-Azido[^{32}P]cAMP. RI and RII were photoaffinity labeled as described by Walter *et al.* (20) in a reaction mixture (80 μl) of 10 mM Mes, pH 6.2/10 mM $MgCl_2$/1.0 μM 8-azido[^{32}P]cAMP containing 100 μg of cytosolic or membrane protein. Where indicated, 100 μM cAMP was included to determine nonspecific labeling. Mixtures were incubated for 60 min in the dark at 4°C and then irradiated for 10 min with a UV lamp. The irradiated samples were pipetted into 20 μl of stop solution [9% $NaDodSO_4$/15% (vol/vol) glycerol/6 mM EDTA/250 mM Tris·HCl, pH 8] and heated at 100°C for 20 sec. Then, 2 μl of 2-mercaptoethanol and 5 μl of 0.1% bromophenol blue in 50% (vol/vol) glycerol were added, and the samples were electrophoresed in 5–10% gradient polyacrylamide slab gels containing $NaDodSO_4$. The gels were dried and autoradiographed at −80°C using Cronex 4 DuPont medical x-ray film. The autoradiographs were scanned in a microdensitometer. When care was taken to not saturate the x-ray film, the peak heights obtained by scanning were proportional to the total radioactivity of the corresponding peaks estimated by scintillation counting as described by Walter *et al.* (20). Levels of RI and RII were calculated by integrating the areas under the curves and subtracting nonspecific labeling.

Score for the Disease. The psoriatic area and severity index (PASI) score has been described by Frederiksson and Petersonn (21). This score takes into consideration the severity of the symptoms of psoriasis (erythema, desquamation, infiltration), rating each on a scale from 0 to 4, and the extent of the disease on four body areas (head, trunk, and upper and lower extremities). The initial formula was slightly modified for our evaluation and was as follows: PASI score = $0.1(Eh + Ih + Dh + Ph)Ah + 0.3(Et + It + Dt + Pt)At + 0.2(Eu + Iu + Du + Pu)Au + 0.4(El + Il + Dl + Pl)Al$, where *E, I, D,* and *P* denote, respectively, *E*rythema, *I*nfiltration, *D*esquamation, and *P*ustules; *h, t, u,* and *l* indicate, respectively, *h*ead, *t*runk, *u*pper extremities, and *l*ower extremities; and *A* is the extent of the disease. A value of 0 indicates no disease, while the value representing maximal severity of the disease is 96.

RESULTS

Binding of 8-Azido[^{32}P]cAMP to RI and RII. To determine possible differences between the protein kinase of normal human fibroblasts and that of psoriatic human fibroblasts, the levels of RI and RII were quantitated by photoaffinity labeling with 8-azido[^{32}P]cAMP. The results presented in Fig. 1 show two bands of specific 8-azido[^{32}P]cAMP binding in cytosol prepared from normal human fibroblasts. The major band (M_r, 51,000) and the second band (M_r, 56,000) correspond to RI and RII, respectively.

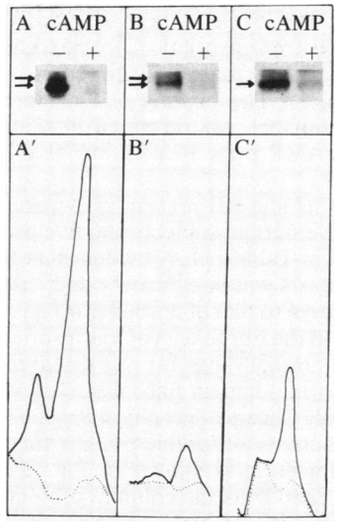

FIG. 1. Specific binding of 8-azido[^{32}P]cAMP to the RI and RII present in cytosol of control and psoriatic human fibroblasts. (*A* and *A′*) The N8 cytosolic fraction from normal fibroblasts. (*B, C, B′,* and *C′*) Cytosolic fractions from fibroblasts of two psoriatic patients. (*A–C*) Autoradiographs showing the photoactivated incorporation of 8-azido[^{32}P]cAMP into cytosolic cAMP binding proteins analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The cytosolic fractions were photoaffinity labeled with 0.1 μM 8-azido[^{32}P]cAMP in the presence (+) or absence (−) of 100 μM cAMP as indicated. One hundred micrograms of cytosolic protein was applied to each gel. (*A′–C′*) Microdensitometric scanning of the autoradiographs. RI and RII levels were determined by subtracting nonspecific labeling (·····) from total area under the curves. Higher molecular weight proteins are on the left.

Table 1. Quantitation of the RI and RII present in cytosol prepared from fibroblasts of normal and psoriatic subjects

Subject	8-Azido[^{32}P]cAMP bound, fmol/mg of protein	
	RI	RII
Normal		
1	1074 ± 151	406 ± 45
2	1207 ± 102	442 ± 20
3	1313 ± 183	505 ± 52
4	1503 ± 101	540 ± 60
5	1625 ± 194	608 ± 64
Psoriatic		
A	851 ± 85	Undetectable
B	546 ± 56	Undetectable
C	155 ± 55	86 ± 50
D	297 ± 41	Undetectable
E	1075 ± 100	214 ± 21

Cytosol was prepared from cultured normal and psoriatic cells. One hundred micrograms of cytosolic protein was photoaffinity labeled with 1 μM 8-azido[^{32}P]cAMP in the presence and absence of 100 μM cAMP and then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The amount of ^{32}P bound was quantitated by densitometric scanning of the radioautograph of the gel. Nonspecific labeling (noted in the presence of added cAMP) was subtracted and levels of RI and RII were calculated based on the specific activity of 8-azido[^{32}P]cAMP. Results represent mean ± SEM of triplicate determinations.

Interestingly, the levels of both RI and RII detected in cytosol prepared from psoriatic fibroblasts of two patients were significantly decreased when compared to normal fibroblasts (Fig. 1). The binding of 8-azido[^{32}P]cAMP was specific; no radiolabel was observed in samples incubated with 100 μM cAMP prior to addition of the photoaffinity label.

The levels of RI and RII present in cytosol prepared from fibroblasts of five normal subjects and five psoriatic subjects are quantitated in Table 1. It is evident that the RII level of psoriatic fibroblasts was significantly decreased, or not even detectable, relative to that of normal fibroblasts. The RI level was decreased in the fibroblasts of four psoriatic patients and was within the normal range for one diseased patient, when compared to normal human fibroblast RI levels.

DE52-Cellulose Chromatography of Protein Kinase Activity. The profile of activity of soluble protein kinase from normal fibroblasts (subject 2) is shown in Fig. 2*A*. Two protein kinases were eluted from the column; protein kinase I eluted between 0.06 and 0.1 M NaCl and protein kinase II eluted between 0.18 and 0.22 M NaCl. The elution pattern of psoriatic fibroblast (subject 6) protein kinase activity was different from that of normal fibroblasts (Fig. 2*B*). The first peak of kinase activity (protein kinase I) eluted at about 0.075 M NaCl. This peak of activity was stimulated by cAMP, although activity in the absence of added cAMP was high. In the region in which protein kinase II was expected little, if any, cAMP-dependent or independent protein kinase activity was detected. The decrease in protein kinase I activity and the disappearance of protein kinase II activity in psoriatic fibroblasts was confirmed by comparison with activity elution profile data for cytosol prepared from fibroblasts of two other psoriatic patients (subjects 7 and 9) (unpublished results). In all cases relatively high cAMP-independent protein kinase activity was observed in the region of protein kinase I, suggesting some dissociation of the enzyme. In all cases the kinase activity profile correlated well with the 8-azido[^{32}P]cAMP binding data.

Quantitation of RI Levels in Normal and Psoriatic Erythrocyte Membranes by 8-Azido[^{32}P]cAMP Photoaffinity Labeling. It has previously been shown that human erythrocyte membranes contain RI but not RII (22, 23). This finding has now been confirmed by densitometric scanning of a radioautograph of specific 8-azido[^{32}P]cAMP labeling of normal erythrocyte membranes (Fig. 3 *A* and *A'*). Similar to results with psoriatic fibroblasts, membranes prepared from erythrocytes obtained from three psoriatic patients (subjects J, K, and B) showed significantly decreased RI levels when compared to

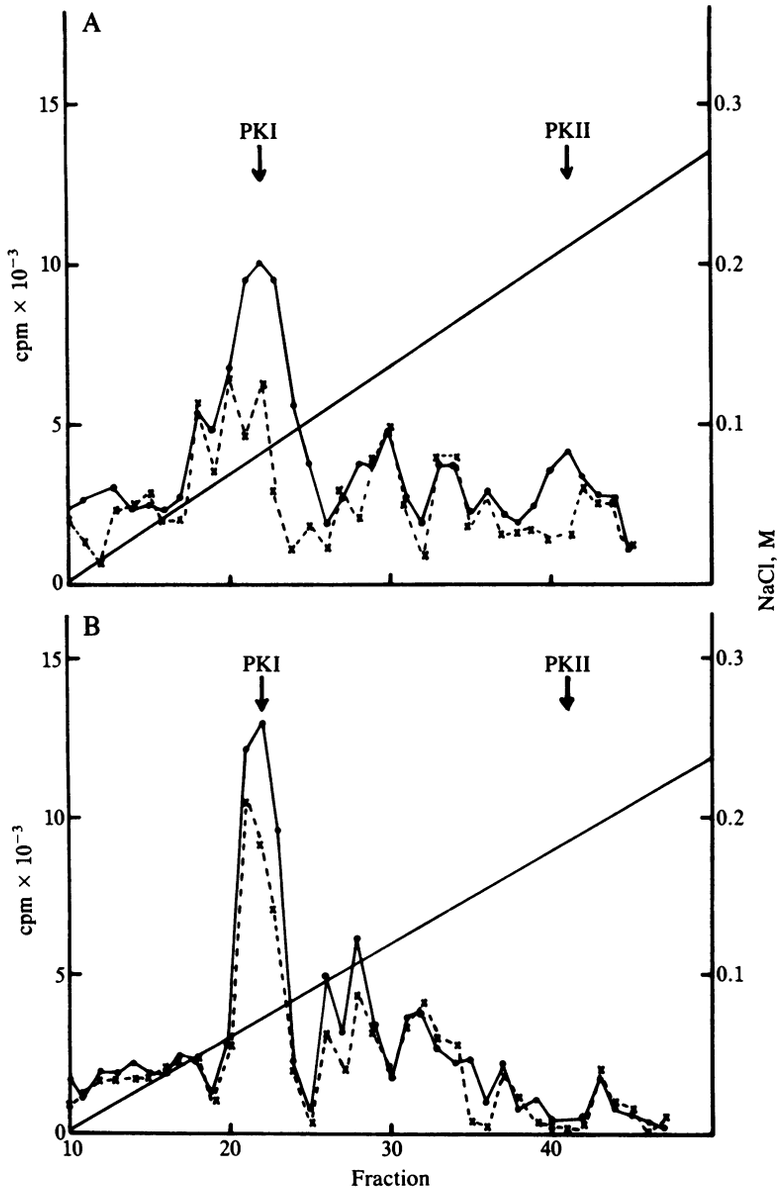

FIG. 2. DEAE-cellulose chromatography of soluble protein kinases. (*A*) Normal fibroblasts (subject 2). (*B*) Psoriatic fibroblasts (subject A). Five-milliliter aliquots of the 50,000 × *g* × 30 min supernatant (20 mg of protein) were chromatographed on a DE52 column in buffer A. Aliquots (30 μl) of the fractions were assayed for protein kinase (PK) activity in the absence (×) or presence (●) of 1 μM cAMP; NaCl concentrations (—) were determined by conductivity.

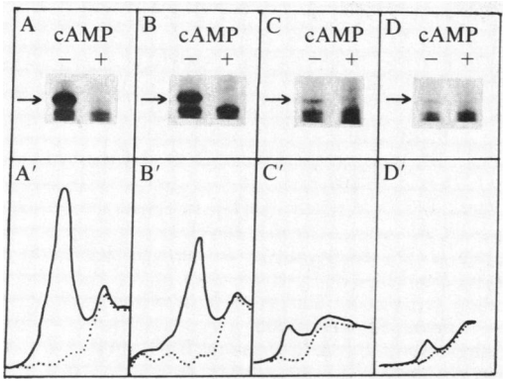

FIG. 3. Specific binding of 8-azido[^{32}P]cAMP to the RI present in erythrocyte membranes from control and psoriatic subjects. (*A* and *A′*) Normal subject (no. 5). (*B–D* and *B′–D′*) Psoriatic subjects J, K, and B. (*A–D*) Autoradiographs showing the photoactivated incorporation of 8-azido[^{32}P]cAMP into erythrocyte membrane cAMP binding proteins analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. One hundred micrograms of membrane protein was applied to each gel. (*A′–D′*) Microdensitometric scanning of the autoradiographs. RI levels were determined by subtracting nonspecific labeling (·····) from total area under the curves.

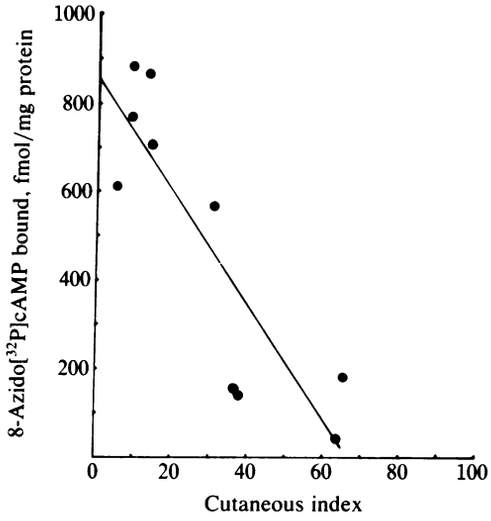

FIG. 4. Correlation between specific binding of 8-azido[^{32}P]-cAMP to the RI present in erythrocyte membranes of psoriatic subjects and severity of the disease of these patients as determined by PASI cutaneous score. $P < 0.001$; $r = 0.872$.

that of normal erythrocyte membranes (Fig. 3 *B–D* and *B′–D′*). Of importance is the observation that the RI level was inversely related to the severity of the disease (as measured by PASI score)—(i.e., subject J, PASI 5.2; subject K, PASI 36.6; subject B, PASI 63.7).

RI levels in erythrocyte membranes obtained from eight normal subjects and 10 psoriatic subjects were quantitated and the results are presented in Table 2. These results indicate that the decrease in RI levels of psoriatic erythrocytes relative to those in normal erythrocytes becomes more pronounced with increased severity of the illness as determined by cutaneous PASI score. It is of considerable interest that a significant negative correlation ($P < 0.001$) was found between the severity of the cutaneous manifestation of the disease and the amount of the RI present in psoriatic erythrocyte membranes (Fig. 4).

Table 2. Quantitation of the RI present in erythrocyte membranes obtained from normal and psoriatic subjects

Subject	RI, fmol of 8-azido[^{32}P]cAMP bound/mg of protein	Cutaneous index
Normal		
5	900–1100	
6	846–1034	
7	716–840	
8	949–1115	
9	965–1125	
10	979–1081	
11	894–1078	
12	512–872	
Psoriatic		
A	511–611	30.8
B	12–72	63.7
F	791–973	9
G	749–789	9
H	781–949	13.3
I	640–772	14.4
J	558–664	5.2
K	138–168	36.6
L	124–156	37.8
M	153–209	65.3

Membranes were prepared from erythrocytes from eight normal and 10 psoriatic subjects. One hundred micrograms of membrane protein was photoaffinity labeled with 1 μM 8-azido[^{32}P]cAMP in the presence and absence of 100 μM cAMP. ^{32}P incorporation was determined as described in the legend to Table 1. Results represent ranges of duplicate determinations.

DISCUSSION

Protein kinases have been implicated in the regulation of cell growth and differentiation, as well as in the viral transformation of cells (7). Abnormal protein kinase activities have been reported in several different cell types, including tumor cells (24) and in mutagenized cells in culture (25, 26). In these cell types the aberrant protein kinases appear to be responsible for the failure of cAMP to induce certain specific enzymes or to regulate cell growth.

Lesional epidermis in psoriasis is characterized by increased cellular proliferation and decreased cellular differentiation (1–3). Previous studies have suggested a possible role for cAMP in mediating epidermal cell proliferation and differentiation (1). Yet, Mier *et al.* (27) have reported normal protein kinase activity in the skin of psoriatic patients.

The hyperproliferation and altered protein synthesis characteristic of psoriasis are also noted in the dermis (28) and in other sites (5) in addition to the skin. Thus we have carried out studies to determine protein kinase levels in cells other than those thought to be specifically involved in this proliferative skin disorder. Of importance is the finding that fibroblasts prepared from psoriatic patients exhibit decreased protein kinase I, and complete loss of protein kinase II, activities, with corresponding changes in levels of RI and RII. In this study, fibroblasts were taken from involved psoriatic skin. More recent studies with three other patients support these results and show that involved and noninvolved psoriatic skin present the same pattern of kinases. Further, the RI level is also decreased in erythrocyte membranes from psoriatic patients when compared to the erythrocyte membrane levels in control subjects. In a related study, an abnormal protein phosphorylation pattern has been described in psoriatic erythrocyte membranes (17).

These results indicate that protein kinase deficiency may be an important indicator of psoriasis severity. This is shown by the significant negative correlation between the disease severity, as determined by cutaneous score, and RI level in psoriatic erythrocyte membranes (Fig. 4). The ease in obtaining erythrocytes from patients and the reliability of quantitative erythrocyte membrane RI levels suggest that

measurement of RI levels may be of clinical importance in establishing the presence, evolution, and severity of the disease.

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