Gastrin-releasing Peptide: In Vivo and in Vitro Growth Effects on an Acinar Pancreatic Carcinoma

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INTRODUCTION

The mammalian gastrin-releasing-peptide (GRP) and its structural amphibian analogue, bombesin, are known to be trophic factors for the normal exocrine pancreas. This work investigates the possible role of GRP in the growth of an acinar pancreatic cancer transplanted to the rat and in primary tumor cell cultures. Moreover, this adenocarcinoma was tested for its content of specific bombesin/GRP receptors by using autodigraphic techniques and in vitro binding assays with tumor cells.

In Lewis rats bearing the pancreatic carcinoma transplanted s.c. in the scapular region, chronic administration of GRP at the dose 30 μg/kg/day for 15 successive days significantly increased the tumor volume, the final tumor weight, and amylose, protein, RNA and DNA contents. Autoradiographic studies showed that tumor tissue was GRP receptor positive with a high density. The biochemical characterization indicated that receptor positive tumor tissue had saturable and high affinity receptors with pharmacological specificity for GRP and its bioactive analogues. In primary tumor cell cultures, GRP increased the incorporation of [3H]thymidine in DNA in a dose- and time-dependent manner. There was a good correlation between the ability of GRP and its COOH terminal analogues to elicit DNA synthesis and their affinity for 3H-GRP binding sites.

These results from in vivo and in vitro experiments demonstrated that GRP induces growth of pancreatic carcinoma by acting directly on specific membrane receptors present on the tumor cells.

MATERIALS AND METHODS

Materials

Lewis rats (180–200 g body weight) were purchased from CNRS Orly (France), and were housed in a vivarium facility maintained at a constant temperature of 25°C. Mimosomic pumps (Alzet, Model 2002) were supplied by W. F. D. A., and were housed in a vivarium facility maintained at a constant temperature of 25°C. Miniosmic pumps (Alzet, Model 2002) were supplied by W. F. D. A.

Experimental Procedures

In Vivo Studies

Lewis rats (180–200g) received in the interscapular region an injection s.c. of 5 to 10 × 10⁶ tumor pancreatic cells in a volume of 0.5 ml. of the known actions of BBS/GRP are mediated by specific receptors. GRP receptors have been characterized pharmacologically on pancreatic acinar cells (29), pituitary cell lines (30), small intestine (31), Swiss mouse 3T3 fibroblasts (32), human small cell lung carcinoma cell lines (33), and a clonal cell line derived from hamster pancreatic islet cells (34).

In a previous work we have reported that chronic administration of BBS or GRP to rats induced pancreatic hypertrophy and hyperplasia (14, 15). The exact role of these peptides in the development and growth of pancreatic cancer is not clearly understood and is still an issue of discussion. Concerning pancreatic carcinogenesis, two animal models can be distinguished. (a) The acinar cell-type adenocarcinoma induced by azaserine in rats and, (b) the ductal/ductular cell type adenocarcinoma induced by nitrosamines in hamsters. It has been shown that BBS enhances the preneoplastic lesions of acinar cell type in rats (35, 36), but exerts contradictory effects on ductular cell-type lesions in hamsters (37). However, little is known about the implication of BBS-LP in growth of pancreatic cancers. In addition, since these peptides generally stimulate the endogenous release of various endocrine secretions which could interfere in vivo with tumor growth, it seems difficult to obtain unambiguous evidence for a direct effect of these peptides. Thus, the aim of this study was to investigate the effects of GRP on growth of an acinar pancreatic tumor transplanted into Lewis rats, as well as in primary cell culture. This model was originally induced by azaserine in the rat. Finally, to further elucidate the actions of this peptide on tumor pancreatic cells, the presence of specific receptors and their relationships with DNA synthesis were investigated.

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The abbreviations used are: BBS-LP, bombesin-like peptides; GRP, gastrin-releasing peptide; NC, neuremoin-C; EGF, epidermal growth factor; HEPES, N-(2-hydroxyethyl)-piperazine-N'-(2-ethanesulfonic acid); BSA, bovine serum albumin; PBS, phosphate-buffered saline; HBSS, Hank's balanced salt solution; FCS, fetal calf serum; MDM, modified Dulbecco's medium; BBS, bombesin; EGTa, ethyleneglycol bis(β-aminoethyl ether)-N,N',N'-tetaacetic acid.
Palpable tumors appeared in 95-100% of rats, after 10-15 days. Twenty Lewis rats, bearing pancreatic tumors, were randomly divided into 2 groups of 10 rats each, receiving either GRP (30 μg/kg/day) or saline (control group), delivered by means of miniosmotic pumps. The tumor volumes were measured with calipers 3 times weekly. The tumor volume was calculated according to the formula

\[ \text{Volume} = \frac{4}{3} \pi \times \text{maximal length} \times \text{maximal height} \times \text{maximal width} \]

assuming an ellipsoid shape. Tumor-doubling times were calculated from semilogarithmic plots of the tumor volume versus time. On day 15, the animals were sacrificed. The tumors and the pancreata were quickly removed and carefully trimmed, weighed, and stored at -20°C. They were homogenized in ice-cold distilled water (100 mg/ml) in a polytron set at medium speed. Protein content was determined by the method of Lowry et al. (38). Amylase content was measured according to the method of Danielsson (39), using maltose as a standard. RNA content was measured by using the orcinol procedure and yeast RNA as standard (40), and DNA content was measured by the diphenylamine procedure, using calf thymus DNA as the standard (41).

2. In Vitro Studies

Primary Pancreatic Tumor Cell Culture. Tumor tissue from s.c. transplanted pancreatic adenocarcinoma was minced, washed extensively in cold phosphate buffer salt Ca²⁺, Mg²⁺-free, and passed through a stainless steel screen (no. 30). The resulting slurry was washed with a large volume of cold HBSS without Ca²⁺/Mg²⁺, and then centrifuged at 150g for 2 min at 4°C. The cells were resuspended in an adequate volume of Dulbecco's modified Eagle's medium supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin) and layered over the Iscove's MDM medium containing 4% BSA. Finally, the cells were dispersed in an enriched medium which consisted of 1:1 (v/v) Iscove's MDM and Waymouth medium supplemented with 15% heat-inactivated FCS, 1 mg/ml BSA, 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 mg/ml Fungizone, 0.25% (w/v) trypsin, 0.02% EDTA, pelleted by centrifugation (3 min at 300xg at 4°C), and resuspended in 2 ml of media. Aliquots were stained with trypan blue and viable cells were counted by a hemacytometer.

For experimental purposes, 2-5 × 10⁵ cells in 2 ml of enriched medium were seeded into individual collagen-coated wells of 6-well plates, and allowed to attach overnight at 37°C prior to experimentation. To study the growth effects of GRP, experiments were conducted in basal medium containing 2.5% FCS and without the addition of hormones or growth factors. The culture was maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C, and the medium was changed every day.

Growth Assessment. The effect of GRP on the growth of tumor cells in culture was determined by direct cell counts and [³H]thymidine incorporation into DNA. After plating and cell attachment overnight, the enriched medium was aspirated and the cultures were washed twice with FCS free-Iscove's MDM medium to remove residual serum. After 24 h of serum starvation, the culture basal medium was supplemented with 2.5% FCS and without the addition of hormones or growth factors. The culture was maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C, and the medium was changed every day.

3. GRP Receptors

Receptor Autoradiography. Visualization of GRP-related peptides in pancreatic tumors was performed by using ¹²⁵I-GRP as radioligand. For autoradiography, frozen tumor fragments were cut into 20-μm thick sections with a cryomicrotome (Microm, Franceville, France) at -20°C and mounted onto gelatin-coated glass slides.

Tissue sections were preincubated in 50 mM Tris-HCl buffer (pH 7.2), containing 2 mM CaCl₂, 5 mM KCl, and 0.5% (w/v) BSA, at ambient temperature for 15 min. Sections were washed twice with 50 mM Tris-HCl buffer without salt and then incubated for 2 h at room temperature with 150 mM Tris-HCl, pH 6.8, containing 120 mM NaCl, 4.7 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 μg/ml leupeptin, 40 μg/ml bacitracin, and 1% BSA, in the presence of 50 μM ¹²⁵I-GRP.

The nonspecific binding was determined by incubating alternate slides with the labeled GRP in the presence of 1 μM concentration of unlabeled GRP. At the end of the incubation, sections were washed twice for 5 min in ice-cold incubation buffer containing 0.5% BSA. Sections were washed twice in ice-cold water, dried quickly, and then exposed to ²H-sensitive films (Amersham France) and exposed for 1 to 3 weeks in radiograph cassettes at -70°C. After revelation of the autoradiograms, the corresponding tissue sections were stained with hematoxylin/eosin.

Receptor-binding Assay. Binding studies were performed in fresh pancreatic tumor cells and in primary cultured cells grown as described above. For binding assays, the medium was discarded and cells were washed twice with PBS buffer, pH 7.4, and then incubated at ambient temperature for 60 min in 50 mM HEPES buffer, pH 7.4, containing 120 mM NaCl, 4.7 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1% (w/v) BSA, 0.01% soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 40 μg/ml bacitracin, with 25 μM ¹²⁵I-GRP in the absence (total binding), or presence (non-specific binding) of 1 μM unlabeled GRP. Incubation was stopped by the addition of 1 ml ice-cold HEPES buffer. The cells were washed twice with ice-cold PBS buffer, scraped, collected, and transferred to Eppendorf tubes containing HEPES buffer supplemented with 4% BSA. The cells were pelleted at 10,000 × g for 2 min and washed twice with ice-cold PBS buffer. The bottoms of the tubes were cut and pellets were counted in a Packard gamma counter at 80% efficiency.

4. Statistical Analysis

The data were presented as means ± SEM. For group comparisons a one-way analysis of variance followed by a Newman-Keuls test was applied; statistical significance was assumed when P < 0.05.

RESULTS

Effect of GRP on Tumor Growth in Lewis Rats. The experiment was started when the tumors became palpable and was terminated after 15 days of GRP treatment. Chronic administration of GRP (30 μg/kg/day) did not influence the growth of the animals and there was no significant difference in body weight gain between the GRP and control groups observed.

Fig. 1 shows that GRP stimulates tumor growth throughout the experiment. During the first 4 days of treatment, no significant difference between the control and the treated group could be found. However, from the fifth day of treatment, the mean tumor volume in the treated group was 38% higher (P < 0.05).
GROWTH EFFECTS OF GRP

Absolute tumor volume

Fig. 1. Effect of GRP on tumor volumes of acinar cell carcinoma transplanted in Lewis rats. The tumor size is plotted as a function of time of treatment. Points, mean tumor volume; bars, SEM. Control group (○) and GRP-treated group (•) were compared at each period. *, P < 0.05; **, P < 0.01.

Fig. 2. Final tumor weight, total amylase, protein, RNA and DNA content in control (ca) and GRP-treated (•) groups after 15-day treatment. **, P < 0.01; ***, P < 0.001.

Table 1 Normal pancreatic weight and its protein, amylase, and RNA and DNA content

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GRP</th>
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</thead>
<tbody>
<tr>
<td>Pancreatic wt (mg)</td>
<td>625.75 ± 25.73</td>
<td>745.70 ± 8.59*</td>
</tr>
<tr>
<td>Amylase (×10^2 units)</td>
<td>140.00 ± 5.00</td>
<td>160.00 ± 5.00*</td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>171.00 ± 6.67</td>
<td>200.00 ± 11.11*</td>
</tr>
<tr>
<td>RNA (mg)</td>
<td>14.50 ± 1.00</td>
<td>20.50 ± 0.50*</td>
</tr>
<tr>
<td>DNA (mg)</td>
<td>2.41 ± 0.05</td>
<td>2.70 ± 0.03*</td>
</tr>
</tbody>
</table>

* P < 0.01.
* P < 0.05.
* P < 0.001.

than in the control group. At the end of the experiment this effect was more pronounced and seemed to stabilize at 60% (P < 0.001) over control values. The tumor-doubling time was reduced by GRP (3 days) in comparison to the control group (4.5 days) as determined from the semilogarithmic growth curves.

Tumor growth was also evaluated by the measurement of tumor weights and biochemical parameters. Fig. 2 shows that GRP treatment increased final tumor weight, protein, and amylase contents by 68% (P < 0.01), 61% (P < 0.001), and 80% (P < 0.001), respectively. RNA and DNA contents increased, respectively, by 31% (P < 0.01) and 40% (P < 0.01).

The growth effects of GRP in the normal pancreas of treated Lewis rats were summarized in Table 1. All parameters measured, i.e., pancreatic weight, and content of protein, DNA, RNA, and amylase increased significantly, but were less pronounced than the effects obtained on tumor growth.

**BBS/GRP Receptor Identification.** Looking for the presence of BBS-like peptide receptors, our results indicate that the pancreatic acinar cell tumor is GRP receptor positive.

The autoradiograms obtained from adjacent sections of pancreatic tumor fragments were incubated with 50 pM 125I-GRP in the absence (Fig. 3A) or presence (Fig. 3C) of an excess of unlabeled GRP, and were compared with the histology of these sections after staining with hematoxylin/eosin (Fig. 3A). We noted that a high density of receptor labeling is principally present in tumor tissue, but not in the surrounding stroma.

In addition to the autoradiographic visualization, the tumors were biochemically characterized. The GRP receptor-binding studies indicated the presence of a single class of high-affinity sites (Kd = 0.23 nM) and a relative high density of sites (Bmax = 113 fmol/mg protein) (Fig. 4B). The pharmacological specificity of binding for GRP was investigated. Fig. 4A shows that the concentration of unlabeled peptide required to inhibit 50% of the specific 125I-GRP-binding activity for GRP, BBS, and NC was approximately 1 nM. In contrast, GRP 1-16 and EGF did not compete with the 125I-GRP-binding sites. Thus bioactive peptides, structurally related to GRP, were able to displace the specific binding while unrelated peptides failed to do the same.

**Effect of GRP on Tumor Cell Growth in Primary Culture.** In preliminary experiments to establish the optimal conditions for tumor cell growth, we found that 0 to 3% of fetal calf serum in the culture medium was not able to induce a significant stimulation of DNA synthesis. Therefore we have chosen to culture pancreatic tumor cells in a basal medium containing 2.5% FCS. In these conditions, the cell population was quite homogeneous without fibroblastic contamination.

When GRP (10^-12 to 10^-6 M) was added to the culture medium 3 times a day during 24 h, it increased the incorporation of [3H]thymidine into DNA in a dose-dependent manner. As illustrated in Fig. 5, this increase was significant (32%; P < 0.05) from 10^-11 M and maximal (84.5%; P < 0.001) at 10^-7 M.

When the primary cultured cells were incubated with GRP for 48 and 96 h, the proliferative effect was more pronounced than after 24 h of treatment (Fig. 6, A and B).

Moreover, DNA synthesis elicited by GRP was markedly potentiated by the addition of 5 μg/ml Insulin and 25 ng/ml EGF (Fig. 6C).

**GRP-Mitogenic Effect Could Be Related to High and Specific GRP-Binding.** To determine whether GRP-mitogenic effect could be related to the high and specific GRP binding, separate experiments were parallely conducted. Tumor cells were grown in the enriched medium and then in basal medium for 24 h repeatedly, and the incorporation of DNA was measured.
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Fig. 3. Distribution of GRP-binding sites in the pancreatic cancer tissue. (A) H&E-stained section; (B) autoradiogram showing total binding of 125I-GRP; (C) non-specific (in presence of 1 nM GRP). The bar represents 2 mm.

before examining the structure-activity relationships, using different peptides structurally related and unrelated to GRP.

Fig. 7 shows that the amphibian BBS analogue, the COOH-terminal GRP 18-27 fragment (NC), as well as the unlabeled GRP 1-27, inhibited the specific 125I-GRP binding in a concentration-dependent manner. Interestingly, when we compared the binding specificity of GRP to the [3H]thymidine incorporation results obtained following the stimulation of the tumor cell growth by the same range of peptides tested for their ability to compete with the labeled GRP, we observed a close parallelism between the relative mitogenic potencies of these peptides and their ability to competitively inhibit specifically bound GRP. The bombesin antagonist [d-Arg1, d-Pro2, d-Trp7,9, Leu11] substance P partially blocks the specific binding of 125I-GRP and has only a slight effect on the stimulation of DNA synthesis by GRP. However, the amino-terminal fragment of GRP (GRP 1-16) neither inhibits 125I-GRP binding nor stimulates DNA synthesis.

DISCUSSION

During the last years, several gastrointestinal hormones and regulatory peptides have been reported to affect the growth of certain gastrointestinal malignancies (42). However, the possible role of hormonal therapy in gastrointestinal and pancreatic cancers has not been fully examined. The BBS-LP are produced by various tumors and these peptides may serve as a hormonal

Fig. 4. Binding of 125I-GRP to the pancreatic tumor cells. A, competitive inhibition of specific 125I-GRP binding by increasing concentrations of unlabeled GRP 1-27 ( ), NC ( ), BBS ( ), GRP 1-16 ( ), and EGF ( ). B, Scatchard plot of the inhibition curve with unlabeled GRP ( ). Cells were incubated with 25 pm 125I-GRP for 30 min at 24°C with varying concentrations of unlabeled peptides. Nonsaturable binding was subtracted from total binding and saturable binding is expressed as the percentage of maximal saturable binding. Points, mean of triplicate determinations from 2 experiments.

Fig. 5. Concentration dependence of GRP on DNA synthesis by the primary pancreatic tumor cell culture. Pancreatic tumor cells (2 x 10⁵) were plated into individual wells of a 6-well plate and incubated for 24 h with enriched medium; then, the cultures were equilibrated in basal medium for 24 h prior to the addition of increasing concentrations of GRP in 2 ml of basal medium containing [3H] thymidine for 24 h. DNA synthesis was assessed by incorporation of radioactivity into acid-insoluble material. Columns, mean of 2 separate experiments performed in triplicate; bars, SEM. Comparisons with control: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
GROWTH EFFECTS OF GRP

Another possible explanation for the inhibitory effect of BBS on the growth of pancreatic ductular adenocarcinomas may be the release of endogenous somatostatin, which has been shown to inhibit tumor growth (44, 45). If so, why would somatostatin not also inhibit the growth of the normal pancreas as it particularly possesses somatostatin receptors (46)? These results are difficult to explain, since the authors did not investigate the presence of BBS receptors in the tumor tissue. The crucial question remains if the inhibition is related to the cell origin, to the cell-differentiation stage, or to a negative growth regulation. It is of paramount importance to develop further studies to clarify this ambiguity and to understand the mechanism of the inhibitory effects of BBS. We cannot exclude that hormones interact differently with atypical acinar cells than with ductular or centroacinar cells.

In our study the trophic effect on the normal pancreas is more likely a direct effect, since (a) BBS/GRP receptors were identified in this tissue; (b) GRP induces a proliferative effect on the acinar cell carcinoma in primary culture; and (c) the combination of BBS with the cholecystokinin-specific receptor antagonist CR 1409 did not modify the trophic response induced by BBS (47). In addition, the direct effect of BBS has been reported in a normal acinar pancreatic culture model (48).

This study investigated the BBS/GRP receptors in pancreatic

Fig. 6. Stimulation of DNA synthesis as a function of time. Tumor pancreatic cells were cultured as described in Fig. 5 and were treated with GRP 3 times daily for 48 h (a) and for 96 h (b), or with GRP and insulin or EGF for 24 h (c). Columns, mean of [3H]thymidine incorporation of triplicate determinations from 2 experiments; bars, SEM. Comparison with control cultures (in absence of GRP): **, P < 0.01; ***, P < 0.001.

marker of such a tumor. Furthermore, by their property of influencing normal pancreatic growth, these peptides may also interfere with the development and growth of pancreatic tumors. The present study is the first report showing that GRP stimulates the azaserine-induced pancreatic cancer growth, both in vivo and in primary culture cells; this model provides a useful experimental system for elucidating the extracellular factors that affect cell proliferation. In fact, GRP treatment at the dose 30 /ig/kg/day delivered by miniosmotic pumps for 15 days induces growth of the transplantable azaserine-induced pancreatic cancer in the rat. The tumor growth was evident in all measured parameters, including tumor volume, weight, protein, amylase, and RNA and DNA contents. Obviously, GRP treatment induced a trophic effect in the normal pancreas, principally due to cellular hypertrophy and hyperplasia, confirming our previous findings (14, 15). If the level of growth stimulation in the normal and tumorous pancreas are compared, the effect on the tumor tissue was more pronounced than in normal tissue. In contrast, Alexander et al. (43) demonstrated that i.p. injections of BBS at the dose 60 /ig/kg/day, inhibited a xenografted pancreatic cancer tissue in nude mice. In addition, Meijers et al. (37) reported that BBS inhibits the development of putative preneoplastic ductular tissue in hamster. The authors explain that these inhibitory effects are related to the cell origin, because these tumors are ductular in origin, while azaserine model is acinar in origin. This hypothesis seems to be in contrast to our previous observation of mitotic figures in both ductular and acinar cells from BBS-treated rats (14).

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Fig. 7. Relationship between receptor binding and DNA synthesis on tumor pancreatic cell culture as a function of the concentration of GRP-related peptides. Top, stimulation of DNA synthesis was performed for 24 h in presence of GRP (O), NC (C), BBS (I), GRP 1-16 (A), and [d-Arg', d-Pro', d-Trp', Leu'] substance P coincubated with 10 nM GRP (D). Point, mean of triplicate samples from 2 representative culture experiments. The values of [3H]thymidine incorporation are expressed as a percentage of the maximum effect obtained with 100 nM of concentration of GRP. Bottom, inhibition of [125I]GRP binding. The cultures were incubated with [125I]-GRP as described in the experimental procedure, in presence of the same range of GRP-related peptides used for DNA synthesis. Binding of [125I]-GRP is expressed as a percentage of the specific binding obtained in the absence of any competitor.
tumor tissue. The autoradiographs indicated that azaserine-induced pancreatic cancer was BBS/GRP receptor positive with a high homogenous density in the tumor tissue. Biochemical and pharmacological characterization of these receptors in tumor cells was established. Scatchard analysis showed the presence of a single class of specific binding sites of high affinity and density ($K_d = 0.23$ nM; $B_{max} = 113$ fmol/mg protein).

Consequently, there is a strong possibility that tumor growth in vivo is mediated via the GPR receptors.

Moreover, the results obtained from the tumor cell culture experiments, supported earlier observations from in vivo studies, indicating that GPR stimulates DNA synthesis. This proliferative effect seems to be concentration, time, and receptor dependent.

Our experimental data indicated that GPR stimulates DNA synthesis of this primary tumor cell culture in the presence of 2.5% FCS. The maximum effect was obtained with 100 nM GRP. This was even markedly potentiated by the addition of insulin and EGF, suggesting that GRP, as well as insulin and EGF, acts on separate receptors. In addition, an important aspect of the study of GPR receptors is the parallelism between the ability of peptides related to GPR (BBS and NC) to bind to GPR receptors, and their capacity to induce DNA synthesis. These data show that the GPR peptide family acts via a distinct signaling mechanism, and eventually leads to a proliferative response. In fact, it is well demonstrated that binding of BBS/GRP to its surface receptors is accompanied by stimulation of phosphoinositol turnover and subsequent mobilization of Ca$^{2+}$ from intracellular stores, and activation of protein kinase C (34, 49, 50).

Our data present the first evidence for direct control of growth of an acinar pancreatic carcinoma by GRP.

We believe that this is compelling evidence that indicates that GRP can indeed be trophic for both normal and neoplastic pancreatic cells, and that this effect is mediated by the interaction of GRP with its receptors. GRP may also in some instances present the first evidence of direct control of growth of an acinar pancreatic carcinoma by GRP.

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