Matrix Metalloproteinase 2 in Tumor Cell-induced Platelet Aggregation: Regulation by Nitric Oxide

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ABSTRACT

A correlation exists between the ability of tumor cells to aggregate platelets and their tendency to metastasize. Tumor cell-induced platelet aggregation (TCIPA) facilitates the embolization of the vasculature with tumor cells and the formation of metastatic foci. It is well documented that matrix metalloproteinases (MMPs) play an integral part in tumor spread and the metastatic cascade. Therefore, we have examined the role of MMPs during TCIPA and its regulation by nitric oxide (NO) in vitro. Human HT-1080 fibrosarcoma and A549 lung epithelial cancer cells induced TCIPA in a concentration-dependent manner that was monitored by aggregometry. This aggregation resulted in the release of MMP-2 from platelets and cancer cells, as measured by zymography. HT-1080 cells released significantly more MMP-2 than A549 cells and were more efficacious in inducing TCIPA. Inhibition of MMP-2 with phenanthroline (1–1000 μM), a synthetic inhibitor of MMPs, and by neutralizing anti-MMP-2 antibody (10 μg/ml) reduced TCIPA induced by HT-1080 cells. TCIPA was abolished by simultaneous inhibition of platelet function with acetylsaliclyc acid (100 μM; thromboxane pathway inhibitor), apyrase (250 μg/ml; ADP pathway inhibitor), and phenanthroline. NO donors such as S-nitroso-n-acetylpenicillamine and S-nitrosoglutathione (both at 0.01–100 μM) inhibited TCIPA and MMP-2 release from platelets and tumor cells. The inhibitory actions of S-nitroso-n-acetylpenicillamine and S-nitrosoglutathione (both at 0.01–100 μM) inhibited TCIPA and MMP-2 release from platelets and tumor cells. The inhibitory actions of S-nitroso-n-acetylpenicillamine and S-nitrosoglutathione were reversed by 1H-[1,2,4]oxadiazole[4,3-d]quinazolin-1-one (0.01–30 μM), a selective inhibitor of the soluble guanylyl cyclase. We conclude that (a) human fibrosarcoma cells aggregate platelets via mechanism(s) that are mediated, in part, by MMP-2; (b) NO inhibits TCIPA, in part, by attenuating the release of MMP-2; and (c) these effects of NO are cGMP-dependent.

INTRODUCTION

For over 120 years, it has been well documented that platelets play an integral role in the hematogenous spread of cancerous cells during the metastatic cascade. In 1865, Armand Trousseau (1) reported a high incidence of venous thrombosis in patients with gastric carcinomas. Subsequent work by Theodor Billroth (2) in 1878 showed that on autopsy, human tumor cells are frequently found in association with thrombi. This led Billroth to propose that the hematogenous spread of cancerous cells may be accomplished by tumor cell-containing thrombi. Because platelet aggregates form one of the main components of such thrombi, many scientists throughout the latter half of the 20th century have investigated tumor cell-platelet interactions. The body of scientific evidence from the last 120 years has undeniably shown that platelet aggregation by tumor cells plays a critical role in the pathology of metastasis. The ability of malignant tumor cells to aggregate platelets, i.e., TCIPA (3, 4), confers a number of advantages to the successful metastasis of a cancer cell. When covered with a coat of platelets, a tumor cell acquires the ability to evade the body’s immune system. Indeed, it has been shown that platelets protect tumors from tumor necrosis factor α-mediated cytotoxicity (5, 6). Another survival advantage for the tumor cell is the tendency for the large tumor-platelet aggregate to embolize the microvasculature at a new extravasation site (7). Furthermore, platelets facilitate the adhesion of tumor cells to the vascular endothelium (8) and release a number of growth factors that can be used by tumor cells for growth (9). Recently, it has been shown that platelets contribute to tumor-induced angiogenesis by releasing angiogenic growth factors such as vascular endothelial growth factor (10–12).

Although the importance of TCIPA for tumor metastasis is clear, the molecular mechanism(s) underlying this process remains unknown. A number of mechanisms capable of mediating TCIPA have been demonstrated. During TCIPA by certain tumors, there is a release of ADP that stimulates platelet receptors and induces aggregation (13). Other tumors stimulate aggregation and subsequently activate the coagulation cascade through the generation of thrombin (13, 14). Furthermore, it has been shown that TCIPA is associated with the production of eicosanoids, such as thromboxane A2 that amplifies platelet aggregation (15). Along with the production of eicosanoids, Steinert et al. (16) have shown that platelet surface glycoprotein αIIβ3, the receptor for fibrinogen, plays an important role in platelet aggregation by tumor cells. Moreover, Oleksowicz et al. (17, 18) have shown that MCF-7 human breast cancer cells express not only platelet αIIβ3 but also glycoprotein Ibα, which is the major platelet receptor that binds the subendothelial protein von Willebrand factor and mediates platelet adhesion to the vascular wall.

Recently, we have described a novel pathway of platelet aggregation that is mediated via the release of MMP-2 from platelets (19). MMP-2 induce platelets and eicosanoids (10-12) have shown that MCF-7 human breast cancer cells express not only platelet αIIβ3 but also glycoprotein Ibα, which is the major platelet receptor that binds the subendothelial protein von Willebrand factor and mediates platelet adhesion to the vascular wall. The aim of our present study was to investigate the role of MMP-2 in TCIPA. In addition, we studied the effects of NO donors SNAP and GSNO on the reactions mediated by MMP-2 during TCIPA.

MATERIALS AND METHODS

Blood Platelets. Blood was collected from healthy volunteers who had not taken any drugs for 14 days before the study. Washed platelet suspensions (2.5 × 1011/ liter) were prepared as described previously (27).

Tumor Cell Culture. Two human tumor cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were cultured as monolayers in 250-ml culture flasks at 37°C in a humidified atmosphere with 5% CO2. They were cultured in 90% DMEM with nonessential amino acids, gentamicin (0.05 mg/ml), penicillin (0.06 mg/ml), GSNO, S-nitrosoglutathione; PGI2, prostacyclin; ODQ, 1H[1,2,4]oxadiazole[4,3-d]quinazolin-1-one; INOS, inducible nitric oxide synthase.

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The abbreviations used are: TCIPA, tumor cell-induced platelet aggregation; MMP, matrix metalloproteinase; NO, nitric oxide; SNAP, S-nitroso-n-acetylpenicillamine; GSNO, S-nitrosoglutathione; PGI2, prostacyclin; ODQ, 1H[1,2,4]oxadiazole[4,3-d]quinazolin-1-one; INOS, inducible nitric oxide synthase.
streptomycin (0.01 mg/ml), and 10% fetal bovine serum. The cells were supplied with fresh medium and subcultured three times each week. Cells were detached from the flasks using EDTA (7 mM) in DMEM with 10% fetal bovine serum and gentle shaking. EDTA was then washed away with Tyrode’s solution, and the cells were resuspended in Tyrode’s solution at a concentration of 10^7 cells/ml. All cell culture reagents were purchased from Sigma (Oakville, Canada).

**Platelet Aggregation.** Washed platelets were preincubated for 2 min at 37°C in a whole blood lumi-aggregometer (Chronolog). Platelet aggregation was then initiated by the addition of HT-1080 or A549 cells (2 × 10^5–2 × 10^6 cells/ml) and monitored by Aggro-Link software. Platelet aggregation was measured as an extent of light transmittance and then expressed as a percentage of maximal stimulus taken at a time point when the maximal stimulus reached 50% transmittance.

**Reagents.** PGI₂, N-Acetyl-Pen-Ary-Gly-Asp-Cys, o-phenanthroline, apyrase, acetylsalicylic acid, SNAP, and GSNO were obtained from Sigma. In some experiments, platelets were aggregated in the presence of neutralizing rabbit polyclonal anti-MMP-2 antibodies (28) or control affinity-purified rabbit IgG (29).

These reagents were incubated with platelets for 2 min before the addition of tumor cells. In experiments where the effects of SNAP and GSNO on tumor cells were examined, these compounds were preincubated with the tumor cells for 1 h at 37°C. Antibodies were preincubated with tumor cells for 2 h at 37°C and then washed out of the medium three times with Tyrode’s solution. ODQ (Alexis, San Diego, CA), an inhibitor of the soluble guanylyl cyclase (30), was preincubated with the platelets in the aggregometer 5 min before the addition of SNAP or GSNO.

**MMP Assay.** The aggregates of platelets and tumor cells were centrifuged at 16,000 × g at room temperature for 2 min, yielding the pellet and releasate. The pellets were then homogenized on ice using a Vibra Sonic sonicator (Sonics & Materials Inc. Danbury, CT). Both the releasate and the pellet homogenate were then stored at −80°C until assayed for the presence of MMPs by zymography. Zymography was performed using 8% SDS-PAGE with copolymerized gelatin (2 mg/ml) as described previously (19, 29).

**Microscopy.** Tumor cell-platelet samples were viewed using phase-contrast microscopy equipped with a Nikon camera.

**Statistics.** Statistics were performed using Graph Pad Software Prism 3.0. All means are reported with SE. One-way ANOVA Tukey-Kramer multiple comparisons test, and paired and unpaired Student’s t tests were performed where appropriate, and a P of less than 0.05 was considered as significant.

**RESULTS**

**Tumor Cell-induced Platelet Aggregation.** HT-1080 and A549 cells were tested for their ability to induce platelet aggregation. When platelets were incubated in the aggregometer for 30 min at 37°C without the addition of tumor cells, no platelet aggregation was detected (Fig. 1). However, both the HT-1080 and A549 cells induced platelet aggregation in a concentration-dependent manner (Fig. 1).

**Effects of Prostacyclin and N-Acetyl-Pen-Ary-Gly-Asp-Cys on TCIPA.** To study whether TCIPA induced by HT-1080 cells could be inhibited by classical inhibitors of aggregation, PGI₂ (0.3–30 nM) was preincubated for 2 min before the addition of cancer cells. Fig. 2 shows that platelet aggregation induced by maximal effective concentrations of HT-1080 cells (2 × 10^5 cells/ml) was inhibited in a concentration-dependent way by PGI₂. Furthermore, N-Acetyl-Pen-Ary-Gly-Asp-Cys, a fibrinogen receptor antagonist (31), also inhibited aggregation in a concentration-dependent manner (Fig. 2).

**MMP-2 Activity in Cancer Cell-Platelet Homogenates and Their Releasates.** To study whether MMP-2 is involved in TCIPA, we assayed its activity by zymography. The M₇, 72,000 gelatinase,
identified as pro-MMP-2 (19, 29), was the major gelatinase detected. In the absence of cancer cells, the activity of \( M_{\text{gelatinase}} \), 72,000 gelatinase in platelets was 158 ± 26 arbitrary units of density/mg protein (Fig. 3A). As the concentration of HT-1080 cells used to induce platelet aggregation increased (from \( 10^4 \) to \( 2.5 \times 10^5 \) cells/ml), there was a significant (\( P < 0.0001; n = 4 \)) decrease in the \( M_{\text{gelatinase}} \), 72,000 gelatinase activity in platelet-cancer cell homogenates (Fig. 3A). Correspondingly, a significant (\( P = 0.0012; n = 4 \)) increase in enzyme activity in the releasate was detected, indicating its release during TCIPA (Fig. 3B). However, when aggregation was induced by higher concentrations of HT-1080 (\( 10^6 \) cells/ml), there was an increase in \( M_{\text{gelatinase}} \), 72,000 gelatinase activity in the homogenate (\( P < 0.01; n = 4 \)).

**Release of MMP-2 from Tumor Cells.** The activity of \( M_{\text{gelatinase}} \), 72,000 gelatinase released during incubation of HT-1080 cells and A549 cells was assayed. Equal numbers of HT-1080 and A549 cells (\( 10^7 \) cells/ml) were incubated for 1 h at 37°C, and the activity of \( M_{\text{gelatinase}} \), 72,000 gelatinase was measured by zymography. Under these conditions, HT-1080 cells secreted significantly more \( M_{\text{gelatinase}} \), 72,000 gelatinase than A549 cells (\( P < 0.0001; n = 3 \); Fig. 4A).

**Release of \( M_{\text{gelatinase}} \), 72,000 Gelatinase Induced by HT-1080 and A549 Cells and Its Effects on Aggregation.** Platelet aggregation was induced by the same number of HT-1080 and A549 cells (\( 10^5 \) cells/ml). HT-1080 cells resulted in the release of greater amounts (\( P = 0.012; n = 4 \)) of \( M_{\text{gelatinase}} \), 72,000 gelatinase than A549 cells during TCIPA (Fig. 4B). Finally, the aggregatory effect of HT-1080 cells was significantly greater than that induced by A549 cells (Fig. 4C).

**Effects of MMP Inhibition on TCIPA.** Incubation of platelets with neutralizing anti-MMP-2 antibody (28), but not with control IgG (each at 10 \( \mu \)g/ml), resulted in a significant (\( P = 0.03; n = 3 \)) inhibition of aggregation induced by HT-1080 cells (Fig. 5A). Furthermore, preincubation of HT-1080 cells for 2 h at 37°C with anti-MMP-2 antibody, but not with control IgG (each \( 10 \mu \)g/ml), significantly (\( P = 0.0195; n = 3 \)) reduced the aggregating effects of cancer cells (Fig. 5B), indicating that MMP-2 expressed by cancer cells contributes to TCIPA. Moreover, a synthetic inhibitor of MMPs phenoanthroline (1–1000 \( \mu \)M; Ref. 19) inhibited aggregation induced by HT-1080 cells in a concentration-dependent manner (Fig. 5C).

**Contributions of Thromboxane-, ADP-, and MMP-2-dependent Pathways of Aggregation to TCIPA.** To test the relative contributions of the different platelet-aggregating pathways to TCIPA induced by the HT-1080 cells, we used inhibitors of three major platelet-aggregating pathways (19). To inhibit thromboxane \( \text{A}_2 \)-, MMP-2-, and ADP-mediated pathways of aggregation, acetylsalicylic acid (100 \( \mu \)M), phenoanthroline (100 \( \mu \)M), and appyrase (250 \( \mu \)g/ml) were used, respectively. Combined inhibitory effects of these compounds abolished TCIPA induced by HT-1080 cells (Fig. 6).

**Effects of SNAP and GSNO on Aggregation and MMP-2 Release during TCIPA.** Because NO is a potent inhibitor of platelet activation and aggregation (23, 24), we tested the effects of SNAP and GSNO (each at 0.01–100 \( \mu \)M) on TCIPA induced by HT-1080 cells. Both SNAP and GSNO inhibited TCIPA, as shown by aggreometry (Fig. 7) and phase-contrast microscopy (Fig. 8). Moreover, inhibition of TCIPA by SNAP (100 \( \mu \)M) and GSNO (100 \( \mu \)M) was accompanied by a significant reduction (\( P = 0.0154 \) (\( n = 5 \)) and \( P < 0.0001 \) (\( n = 5 \)), respectively) in the release of \( M_{\text{gelatinase}} \), 72,000 gelatinase during TCIPA (Fig. 9A). Finally, in the absence of platelets, both SNAP and GSNO (100 \( \mu \)M) inhibited (\( P = 0.0196 \) (\( n = 12 \)) and \( P = 0.0037 \) (\( n = 12 \)), respectively) the release of \( M_{\text{gelatinase}} \), 72,000 gelatinase from HT-1080 cells after being incubated with HT-1080 cells for 1 h at 37°C (Fig. 9B).

**Effects of ODQ on TCIPA and MMP-2 Release during Inhibition of Aggregation Induced by SNAP and GSNO.** Finally, we investigated whether the inhibitory effects of NO on MMP-2 release during TCIPA were dependent on the production of cyclic GMP. ODQ, a selective inhibitor of the soluble guanylyl cyclase (30), reversed the inhibition of TCIPA by SNAP (Fig. 10, A and B). Moreover, ODQ (30 \( \mu \)M) also significantly reversed the decrease in \( M_{\text{gelatinase}} \), 72,000 gelatinase release caused by SNAP and GSNO (both at 100 \( \mu \)M; \( P = 0.0359 \) (\( n = 3 \)) and \( P = 0.0032 \) (\( n = 4 \)), respectively) during TCIPA (Fig. 10, C and D).

**DISCUSSION**

The objective of our investigation was to study the role of MMPs in TCIPA, focusing on a novel mediator of platelet aggregation,
MMP-2 (19). Furthermore, we investigated the effects of NO, a known inhibitor of platelet aggregation (23, 24) and TCIPA (25), on the release and the biological activity of MMP-2.

We used two human tumor cell lines, HT-1080 fibrosarcoma and A549 lung epithelial carcinoma, to study the role of MMP-2 in TCIPA. HT-1080 cells were found to be more potent inducers of platelet aggregation than A549 cells. The fact that the A549 cells are not potent stimulators of platelet aggregation is consistent with the results obtained by Heinmoller et al. (32). Furthermore, we found that HT-1080 cells secreted more MMP-2 than the A549 cells. Therefore, the majority of the remaining experiments studying the biological role of MMP-2 in TCIPA and its interactions with NO were carried out using HT-1080 cells.

First, we showed that the interactions between HT-1080 cells and platelets led to aggregation, as evidenced by phase-contrast microscopy and the inhibition of TCIPA by PGI$_2$. PGI$_2$ is the most potent known inhibitor of platelet aggregation (33). Moreover, PGI$_2$ is known to inhibit TCIPA induced by other tumor cell lines (34).

Having established that the interactions between HT-1080 cells and platelets result in aggregation, we studied the role of MMP-2 in TCIPA. We found that aggregation induced by HT-1080 cells was associated with the release of $M_7$, 72,000 gelatinase, suggesting that MMP-2, in addition to agonist-induced aggregation (19, 29), contributes to TCIPA. Several lines of evidence support this hypothesis. First, the release of this enzyme during TCIPA induced by HT-1080 cells was concentration dependent. Second, phenanthroline, a MMP inhibitor that inhibits agonist-induced platelet aggregation (19), reduced TCIPA. Third, TCIPA was significantly decreased in the presence of neutralizing anti-MMP-2 antibody, but not by control IgG. Moreover, the HT-1080 cells that had been preincubated for 2 h with this antibody, but not with control IgG, showed a decreased ability to induce TCIPA. Because anti-MMP-2 antibody was washed out of the medium of HT-1080 cells in these experiments, its aggregation-inhibitory effects are clearly associated with the neutralization of MMP-2 expressed at the surface of the HT-1080 cell membrane. Thus, both platelets and cancer cells may contribute to the MMP-2 pool involved in TCIPA.

The mechanism(s) of the proaggregating actions of MMP-2 are now being elucidated. We have recently shown that gelatinase B (MMP-9) may counteract the proaggregating effects of MMP-2 by inhibiting platelet aggregation (35). However, TCIPA was inhibited by phenanthroline, which inhibits both MMP-2 and MMP-9 activities (35). Therefore, it is likely that MMP-2 is the dominant platelet-regulating gelatinase under these conditions.

The MMP-2-dependent pathway of aggregation triggered by HT-1080 cells interacts with thromboxane and ADP-mediated pathways as revealed by experiments using selective inhibitors of these pathways of platelet aggregation. Thus, similar to agonist-induced platelet aggregation (19), the major pathways of aggregation interact to stimulate TCIPA.

We have previously shown that platelet MMP-2 is translocated during aggregation to the platelet surface membrane, and we proposed that the reactions of MMP-2 with platelet integrin receptors mediate aggregation (29). Moreover, aggregation induced by HT-1080 cells was inhibited by the antagonist of the fibrinogen receptor N-Acetyl-Pen-Arg-Gly-Asp-Cys, indicating that the expression of $\alpha$$_{IIb}$$\beta$$_3$ integrin receptor is a common pathway mediating TCIPA. Furthermore, Brooks et al. (36) have recently shown that MMP-2 is localized to the surface of invasive cells with the integrin $\alpha$$_{IIb}$$\beta$$_3$, thereby facilitating directed cellular invasion. Therefore, we propose that the interactions between platelet and cancer cell surface integrins and MMP-2 are important in mediating the aggregating effects of this MMP during TCIPA.

In addition to degradation of the cellular basement membrane and stimulation of TCIPA, MMP-2 also exerts some vascular effects that could contribute to carcinogenesis. We have recently shown that MMP-2 cleaves big endothelin-1 to yield a novel vasoactive peptide medium endothelin-1 (37). Endothelin-1 is a vasoconstrictor peptide that is known to affect tumor cell transduction mechanisms and stimulate tumor growth (38–41).

The role of NO in cancer growth, invasion, and metastasis has been studied extensively. However, there has been considerable controversy in the literature regarding whether NO promotes or inhibits cancer growth, invasion, and metastasis. This is not entirely surprising, considering the complex and multifaceted actions of NO including regulation of vasodilatation (42) and cell adhesion (43, 44), and its effects on cellular growth, proliferation, and cell migration (45–47). Some studies have demonstrated that NO may promote tumor growth. Mortensen et al. (48) have shown that inhibition of MCF-7 endothelial nitric oxide synthase activity resulted in cancer cell apoptosis. Furthermore, the NOS substrate l-arginine stimulated in vitro bladder carcinoma growth in a dose-dependent manner, an effect reduced by nitric oxide synthase inhibition (49). Moreover, NO may stimulate tumor growth by causing increased tumor neovascularization and...
blood flow. Indeed, three recent studies report that NO plays an important role in mediating tumor-induced angiogenesis (50–53). However, other researchers found that NO can decrease the rate of carcinogenesis. Indeed, high levels of endothelial nitric oxide synthase in microvessels around breast cancers were associated with increased patient survival (54). Moreover, other studies have shown that human colon carcinoma cells isolated from metastases exhibited lower NO activity than cells isolated from the primary tumor and that the metastatic cells were more potent inducers of platelet aggregation (25). Furthermore, it has been established that an inverse correlation exists between the expression of endogenous iNOS and NO production by metastatic cells and their metastatic potential (26, 55–57). In addition, it has been shown that induction of iNOS expression by cytokines such as tumor necrosis factor α and IFN-β induced tumor apoptosis and inhibited tumor growth (58, 59). Interestingly, Amb et al. (60) have shown that induction of iNOS in tumor cells with wild-type p53 genes resulted in inhibition of tumor growth, but iNOS expression in tumor cells with mutant p53 resulted in increased tumor growth, VEGF expression, and neovascularization of the tumor. Thus, NO may either promote tumor growth or be tumoricidal, depending on a number of factors such as the differentiation state of the tumor, its genetic status, vascularization, activation of tumor adhesion receptors, and the concentrations of NO in the tumor microenvironment.

In these experiments, the NO donors, SNAP and GSNO, inhibited platelet aggregation induced by HT-1080 cells in a concentration-dependent manner. Furthermore, these compounds inhibited the release of MMP-2 during TCIPA. Thus, this ability of NO to inhibit MMP-2 release shows that there is a “cross-talk” between NO and MMP-2 during TCIPA. Interestingly, SNAP and GSNO also inhibited the release of MMP-2 from HT-1080 cells. These results have clinical implications because they show that NO donors not only inhibit TCIPA but also reduce the release of MMPs from invasive tumor cells. Moreover, some SNAP derivatives such as glucose-2-SNAP and fructose-2-SNAP are also cytotoxic to cancerous cells (61, 62).

We postulate that the use of NO as a therapeutic tool in inhibiting TCIPA and thereby impeding the metastatic process would require platelet-specific NO donors. In fact, platelet-nonspecific NO donors such as organic nitrates (63) could potentially promote carcinogenesis by causing vasodilatation and increasing blood flow to a growing tumor. Interestingly, GSNO has been shown to be a relatively platelet-specific NO donor because it inhibits platelet aggregation at concentrations that do not cause significant vasodilatation (64). The mechanisms of these effects of GSNO are relatively platelet specific, requiring extracellular enzymatic metabolism of the GSNO molecule (64, 65).

To determine whether the effects of NO on TCIPA were mediated by cyclic GMP or not, we used ODQ, a selective inhibitor of the soluble guanylyl cyclase (30). ODQ was able to reverse the inhibition of TCIPA by the NO donors SNAP and GSNO in a concentration-dependent manner. These results demonstrate that inhibition of TCIPA by NO is cyclic GMP dependent. In addition, ODQ was able
to abolish the inhibition of MMP-2 release from platelets by SNAP and GSNO during TCIPA. Thus, both inhibition of TCIPA and the release of MMP-2 are controlled by cyclic GMP.

In conclusion, we have shown that aggregation of platelets by fibrosarcoma cells HT-1080 depends, in part, on the release of MMP-2 from platelets and cancer cells. The activation of TCIPA by MMP-2 is regulated by NO and cyclic GMP. The clinical significance of these findings remains to be studied.

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