Photoaffinity Labeling of a Cell Surface Polyamine Binding Protein*

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Intracellular polyamine pools are partially maintained by an active transport apparatus that is specific for and regulated by polyamines. Although mammalian transport activity has been characterized by kinetic studies, the actual protein itself has yet to be identified, purified, or cloned. As one approach to this problem, we attempted photoaffinity labeling of plasma membrane proteins using two specifically designed and synthesized polyamine conjugates as photoprobe. The first is a spermine conjugate bearing the photoreactive moiety 4-azidosalicylic acid at the N⁴ position via an alkyl linkage, and the second is a norspermine conjugate with 4-azidosalicylic acid at the N¹ position via an acyl linkage. Labeling of murine L1210 lymphocytic leukemia cells was carried out at 4 °C to promote selective alkylation of cell surface proteins. Separation of plasma membrane proteins from cells cross-linked with the N⁴-spermidine conjugate by SDS-polyacrylamide gel electrophoresis revealed two heavily labeled proteins at ~118 and ~50 kDa (designated p118 and p50, respectively). Band p118 was more well defined and much more intensely labeled. Analogous proteins were also observed in human U937 lymphoma cells. Specificity of labeling was strongly suggested by competition with polyamines and analogs during labeling and further indicated by the nearly identical labeling of the same protein by the N¹-norspermine photoconjugate but not by the unconjugated photoreagent. Neuraminidase pretreatment of L1210 cells increased mobility of the p118, suggesting that it was glycosylated and, thus, a plasma membrane origin. In transport-deficient L1210 cells, p118 and p50 were found to have a slightly higher molecular mass and were accompanied by a less distinct protein band (~100 kDa). These findings indicate the presence of a polyamine binding protein at the surface of murine and human leukemia cells, which could be directly or indirectly related to the polyamine transport apparatus.

Cell proliferation is critically dependent on a constant supply of the intracellular polyamines: putrescine, spermidine (Spd), and spermine (Spm). Under in vivo conditions, this supply is thought to be maintained by sensitively regulated processes involving polyamine biosynthesis, catabolism, and transport. While the key enzymes responsible for biosynthesis and catabolism of polyamines have been biochemically purified, characterized, and cloned, the putative proteins responsible for polyamine transport in mammalian systems have, thus far, only been studied at the functional level.

From kinetic studies, polyamine transport is known to be an active process that is energy- and temperature-dependent and adheres to Michaelis-Menten kinetics (1-3). Although the transporter is highly specific for polyamines, various synthetic molecules such as polyamine analogs, methylglyoxal-bis(guanylhydrazone) (MGBG), and paraquat utilize the carrier as a primary means for gaining entry into cells (4). By contributing to their intracellular accumulation, the transporter undoubtedly determines their in vitro and in vivo pharmacological behavior and hence their therapeutic potential.

Like polyamine biosynthesis, polyamine transport is sensitively and negatively regulated by intracellular pools. Polyamine depletion results in increases in uptake, while polyamine excess causes decreases in transport (5-7). In the absence of appropriate molecular probes, the basis for regulation of polyamine transport remains uncertain. Recent findings suggest that, like the polyamine biosynthetic enzyme ornithine decarboxylase, transport may be at least partially regulated by antizyme, which apparently contributes to the regulated degradation of these proteins (8, 9). In order to investigate such possibilities more directly, the mammalian transporter needs to be purified and/or cloned.

Due to the relatively weak ionic interactions between polyamines and their receptors, standard means of isolation and purification such as affinity chromatography have not proved useful in such systems. Efforts to clone the protein have included cross-species transfection of DNA from transport-positive cells into transport-negative cells as has been done with the proteins involved in folate transport (10). Efforts by Byers et al. (11) led to the transferal of polyamine transport activity by this means but not to the isolation of gene sequences encoding the transporter. In contrast to studies in mammalian systems, Igarashi and co-workers (12-14) have used this approach in bacterial systems to isolate the genes encoding multiple polyamine transporters of Escherichia coli.

In this study, photoaffinity labeling was attempted in an effort to identify and characterize cell surface proteins in mammalian systems that bind polyamines and may be involved in

ASA, N-hydroxysuccinimidyl-4-azidosalicylic acid; p118, predominant plasma membrane protein labeled with polyamine photoprobe at 118 kDa; p50, plasma membrane protein labeled with polyamine photoprobe at 50 kDa; TLC, thin layer chromatography; MGBG, methylglyoxal-bis guanylhydrazone.

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polyamine transport. The approach allows for the covalent modification of specific protein sites by the activation of a light-labile moiety affixed to a particular ligand. First proposed by Ji (15), photolabeling has proved useful in the identification of various plasma membrane transporters and surface receptors (16, 17). Under conditions designed to optimize selective interaction with plasma membrane proteins, custom-synthesized polyamine photoprobe (Fig. 1) were used to modify covalently putative polyamine binding sites. This approach led to the identification of a plasma membrane polyamine binding protein having a molecular mass of ~118 kDa and demonstrating high specific activity of labeling with the polyamine photoprobe; its relevance to polyamine transport is uncertain. These studies have been previously presented in abstract form (18).

**EXPERIMENTAL PROCEDURES**

Materials—MGBG and Spd were purchased as hydrochloride salts from Sigma. Dr. R. Bergeron (University of Florida, Gainesville) kindly supplied the polyamine analog N^2,N^8-dihydroxypermine (DDEpm). The photoactive reagent used in control studies, N-hydroxy succinimidyl-4-azido-3,4-di(2-mesitylene)amine (NHS-ASA) was prepared through a slightly modified procedure (Pierce). NHS dioxanes were purchased from Dupont NEN in the amount of 1 ml. The radionuclide Na^22 was obtained in nonreducing form with a pH range of 8–10. Vibriobacterium neumannii was purchased from Boehringer Mannheim.

Cell Culture—Parental L1210 murine lymphocytic leukemia cells and a subline (L1210/MG) made polyamine transport-deficient by chronic exposure to MGBG (19) were kindly provided by Dr. G. Hiey (University of Umea, Sweden). Human U937 lymphoma cells were acquired through American Type Cell Culture (Rockville, MD). Murine L1210 cells and human U937 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 60 units/ml penicillin, 60 mg/ml streptomycin (Life Technologies, Inc.) and 50 μM β-mercaptoethanol (Sigma). RPMI 1640 was used in the photolabelling studies. NHS-ASA (27) was used as photoreagent and was supplied to a density of 2 × 10^8 cells/ml in phosphate-buffered saline containing 0.1 g/liter CaCl_2 and 2 g/liter glucose and treated with the commercially available reagent NHS-ASA (27). Mycoplasma tests (GenProbe Kit, GenProbe, San Diego, CA) were carried out periodically to ensure contamination-free cultures. For the purpose of reverse phase chromatography, octadecyl columns were obtained from J. T. Baker (Phillipsburg, Nj) were prepared as per the manufacturer’s recommendations.

Iodination of the Photoprobe—Radiiodination of the photoprobe was carried out with 125I-iodo-deoxy-Beads iodination reagent (Pierce) in the dark under a red safety light filter at 4°C to prevent premature photolysis. Removal of the photoprobe, by the method described for the exposure of the photoprobe with 2 μl on fluorescent thin layer chromatography paper and viewing with a short wavelength lamp. The MeOH/HCl eluate was the only positively fluorescent spot. The sample was applied to a silica gel plate of nitrogen gas and not boiled in sodium phosphate buffer. Each of the eluates and the final redissolved sample was counted in a Beckman scintillation counter model LS 8000.

Synthesis of Photoprobe—Photoprobe synthesis of the photoprobe N^4-ASA-Spd began with N^2,N^8-bis(Boc)-4-benzylspermidine prepared according to Bergeron and Garlich (20). This was debenzylated to N^1,N^8-bis(Boc)-4-(2-phthalimidoethyl)spermidine prepared according to (21) by treating it with 10% methanol/ethanol and 10% acetic acid, the azidosalicylic acid (ASA) moiety was affixed via an alkyl linkage, the Spd moiety remaining free of alkyl conjugates (Figs. 1 and 2).

The synthesis of N^4-ASA-nSpm began with the commercially available intermediate 1-benzylmido-4-(N-benzyl-7-{[2-mesitylene)sulfonyl]amino}-4-aza-heptane (22) in the presence of sodium hydroxide to afford a heterogeneously tetraprotected intermediate possessing the requisite nospore backbone. In order to preserve the amide linkage in the intermediate precursor to the target photoprobe, it was necessary to manipulate the nitrogen-protecting groups early in the synthesis to produce an intermediate that could be deprotected under mild conditions. Thus, the nospore protecting group was removed by treatment with triﬂuoroacetic acid (23), and the resulting secondary nitrogen was reprotected as the corresponding N-(p-nitrobenzyl)-2 (21). The phthalimide was then removed by hydrazinolysis (24), and the resulting primary amine was converted to the corresponding N-Boc derivative (25). The N-benzyl- and N-(p-nitrobenzyl)-protecting groups were simultaneously removed by hydrolysis (26) to afford a nospore derivative in which one of the two primary nitrogens was protected. The primary nitrogen in this intermediate was then reacted regioselectively with the commercially available reagent NHS-ASA (27), yielding the penultimate precursor to the target photoprobe. This intermediate, as well as the target molecule, were protected from light at all times to avoid premature photolysis. Removal of the N-Boc protecting group was then accomplished by the method described.

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early structure function studies involving polyamine uptake (32, 33) have shown that derivatization at the N1 position. Thus, a Spd photoprobe was designed and synthesized in which the photoreactive group ASA was affixed to the N4-alkyl linkage is better tolerated than derivatization at the N1 position. Therefore, a Spd photoprobe was designed and synthesized in which the photoreactive group ASA was affixed to the N4 position of Spd via a 2-carbon alkyl bridge (Fig. 1). The purity of the compound was assessed via elemental analysis and thin layer chromatography and found to be 89.7%. The ability of N4-ASA-Spd photoprobe to compete with Spd for transport into murine L1210 leukemia cells was examined by Michaelis-Menten kinetic parameters for uptake. With an apparent $K_i$ of 52 $\mu M$, the photoprobe was found to be within the range of other compounds that utilize the polyamine transport system including MGBG, which competes in these cells with an apparent $K_i$ of 71 $\mu M$ (Table I). Since photoaffinity probes with binding constants in the low micromolar range have been used successfully in the labeling of plasma membrane proteins such as a folate binding protein (28), we presumed that the N4-Spd conjugate could be similarly useful in photocross-linking of polyamine binding proteins at the cell surface.

Previous plasma membrane labeling protocols (34) have established that membrane cross-linking should be carried out at 4°C in order to minimize probe internalization and restrict labeling to the cell surface. To confirm this strategy, accumulation of radioactivity was monitored at 4°C in order to conserve photoprobe, labeling was confined at 35 $\mu M$ unless otherwise indicated.

Separation of proteins contained in plasma membrane preparations of photolabeled cells was achieved by SDS-polyacrylamide gel electrophoresis. Labeled proteins were then identified by autoradiography following exposure of 5–14 days. By Coomassie Blue staining, >60 separate protein bands were visible (Fig. 3). Of these, several were found by autoradiography to be diffusely labeled to varying degrees of intensity. Two, however, demonstrated a high specific activity of labeling, and both were found by Coomassie staining to represent relatively minor membrane proteins. The first was a prominent well-defined band at 118 kDa, and the second was a broader, more diffuse band at 50 kDa (Fig. 3). We have designated the former p118 and the latter p50.

To examine the possible relevance of p118 to polyamine transport activity, the plasma membrane protein labeling pattern in polyamine transport-deficient L1210/MG cells (19) was compared with that of parental L1210 cells. In agreement with the original studies by Persson et al. (19), L1210/MG cells were found to be ~1000-fold resistant to MGBG and to accumulate much less radionucleotides Spd than the parental cells (data not shown). By Coomassie Blue staining, no obvious difference in stained protein pattern was apparent between L1210 and L1210/MG cells (data not shown). Following autoradiography, the photoprobe was competed with radiolabeled Spd for transport into murine L1210 leukemia cells by a photoaffinity labeling method (Fig. 4, lanes 1 and 3). It was observed that the primary labeled protein, p118, consistently exhibited a slightly higher molecular mass form in the L1210/MG cells, and the labeling of a minor band (100 kDa) was more apparent. Even following prolonged autoradiographic exposure (data not shown), no other differences in labeled protein bands were apparent between the parent and

![Structure of photoprobe N4-ASA-[125I]-Spd](image)

**Fig. 1. Structure of photoprob...**

internalization of probe. Samples were then rapidly transferred to one well of a six-well plate and incubated on ice for 5 min, after which cells were exposed to uv light for 3 min. The light source was a Mineralight (Gabriel, CA) 115 V, 50 c.cyc. The plate containing cells was positioned approximately 5 cm from the wide range (254–320 nm) filter. Immediately following uv cross-linking 100 $\mu M$ dithiobiotin was added to scavenge unreacted probe (29). Samples were washed with phosphate-buffered saline, centrifuged, and suspended in a buffer comprising $0.05 M$ boric acid, $0.15 M$ NaCl, $1 \text{mM} MgCl}_2, 1 \text{mM} CaCl}_2,$ and a mixture of protease inhibitors ($10 \mu g/ml of leupeptin, aprotinin, and 10 $\mu g$ phenylmethylsulfonyl fluoride (Sigma)). Following hand homogenization of labeled cells, plasma membranes were isolated by sucrose fractionation according to the procedure of Thom et al. (30). Final membrane preparations were solubilized in 0.5% Triton X-100 phosphate-buffered saline.

Protein Gel Electrophoresis—Plasma membrane proteins were separated on a 12% SDS-polyacrylamide gel according to the procedure of Laemmli (31). Following electrophoresis, the gels were fixed and stained with 0.25% Coomassie Blue in $50\%$ methanol, $7\%$ acetic acid. The gel was then dried and exposed to Kodak X-Omat-AR film with Dupont intensifying screen for 5–14 days.

Photoprobe Accumulation—Cells were washed in phosphate-buffered saline and pelleted twice before resuspension in prewarmed phosphate-buffered saline containing 1% gelatin $CaCl}_2$ and 2% gelatin glucose. Treatment with photoprobes at 2.5 $\mu M$ was performed in the dark under red safety light filter. Triplelicate samples were incubated at 37°C for 30 min while a fourth sample was left on ice as a subtraction control for passive uptake into intact cells at 37°C, virtually none was accumulated at 4°C (Fig. 2). In accordance with previous protocols, all photoaffinity labeling was done at 4°C. In preliminary studies, concentrations of photoprobe at 100 $\mu M$ were found to be saturating (data not shown). In order to conserve photoprobe, labeling was conducted at 35 $\mu M$ unless otherwise indicated.

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![Table 1: Spermidine transport kinetics in L1210 cells](image)

**Table 1:**

| Substrate or competitor | V_max | $K_i$ | $K_i$ |
|-------------------------|-------|------|------|
| Spd                     | 53    | 1.0  | 1.0  |
| MGBG                    |       |      |      |
| DEspm                   |       |      |      |
| N4-ASA-Spd              |       |      |      |
| N4-ASA-nSpm             |       |      |      |
| N5H5-ASA                |       |      |      |

All competitors were tested at 100 $\mu M$ except for DEspm, which was tested at 10 $\mu M$. *Data represent mean values from three experiments run in duplicate or triplicate with little significant difference (~15%). *Apparent inhibitor constants are a representative result; duplicate experiments run in triplicate revealed little significant difference (~15%).
transport-deficient lines.

Neuraminidase pretreatment was used to examine whether negatively charged sialic acid residues contributed to p118 labeling and to compare the glycosylation status of labeled proteins in L1210 and L1210/MG cells (Fig. 4, lanes 2 and 4). This treatment failed to diminish labeling intensity, suggesting that sialic acid was not responsible for photoprobe binding. It did, however, produce a clear increase in mobility of p118 in both L1210 and L1210/MG cells, indicating that the protein was similarly glycosylated in both cell types.

The specificity of p118 as a possible polyamine binding protein was evaluated by first comparing labeling with the unconjugated photoaffinity reagent NHS-ASA. In uptake studies, NHS-ASA was ineffective in competing with [3H]Spd for uptake into L1210 cells (Kᵢ 100 μM). When the unconjugated probe NHS-ASA-[125I] was used to photolabel cells, the protein banding patterns were significantly different from those obtained with N⁴-ASA-[125I]Spd (Fig. 5). In particular, neither p118 nor p50 were significantly labeled, thus suggesting that the polyamine ligand of the photoprobe strongly influenced targeting specificity to sites on the plasma membrane.

The labeling pattern was also compared with another photoaffinity reagent, N¹-ASA-[125I]nSpm, which was synthesized in order to further evaluate the binding specificity of p118. With this conjugate, the ASA moiety is affixed via an acyl bond to the N¹ of the polyamine so that the remaining three charged nitrogens of nSpm presumably are available to act as the binding determinants (Fig. 1). In uptake studies, the N¹-nSpm conjugate competed with [3H]Spd transport more effectively than N⁴-ASA-Spd, with an apparent Kᵢ value of 23 μM (Table I). Thus, N¹-ASA-nSpm was a potentially more specific photoaffinity reagent than the N⁴-conjugate. Although we demonstrated that N⁴-ASA-Spd and especially N¹-ASA-nSpm competed with [3H]Spd for polyamine transport, these data do not indicate the ability of the conjugate to actually utilize the transporter to enter cells. To test this possibility, L1210 and L1210/MG cells were incubated with either of the iodinated conjugates in the dark for 30 min, washed, and then processed for radioactivity content. A clear difference in uptake of the two polyamine photoprobes was seen between the two cell lines, where normal L1210 cells accumulated much more of either photoprobes than the transport-deficient cells (Fig. 6). Accumulation of radioactivity by transport-deficient cells suggests that in addition to utilizing the polyamine transporter, the photoprobes may, to a lesser degree, enter cells via other means, such as passive diffusion.

Photoaffinity tagging of L1210 cells with N¹-ASA-[125I]nSpm resulted in labeling of p118 that was identical to that seen with N⁴-ASA-[125I]Spd (Fig. 7). Thus, a photoprobe that exhibits a
2-fold higher affinity for the transporter also intensely labels p118. The more potent binding affinity of the N1-nSpm photo-probe seemed to be reflected in higher intensity of protein labeling, which was apparent in the shorter exposure times required for autoradiography. Photolabeling of cells with the N1-nSpm probe also resulted in a band at >200 kDa. A similar but more faintly labeled protein was occasionally seen with the N4-Spd probe. Additional studies using a 7.5% gel led to the conclusion that the apparent 200-kDa band was labeled protein that had precipitated at the stack:separating gel interface (data not shown). Comparison of labeling patterns for L1210 and L1210/MG cells tagged with the N1-nSpm probe failed to yield any differences beyond those seen with the N4-Spd probe.

Specificity of p118 labeling was also tested by competition assays whereby excess amounts of polyamines or polyamine analogs were present during the cross-linking reaction. In a series of such assays, cells were incubated with competing Spd, Spm, DESpm, or the noniodinated probe for 10 min before and during exposure to N4-ASA-[125I]Spd. A dose-dependent decline in labeling was apparent (Fig. 8). At 100-1000-fold concentrations, both the natural polyamines and the polyamine analog, DESpm, competed effectively with p118 labeling, reducing the covalent cross-linking by as much as 75%. As per expectations, N4-ASA-Spd was the most potent competitor, reducing labeling at only 10-fold excess. It should be noted that during uv exposure, N4-ASA-Spd binding is covalent and irreversible, and therefore not subject to reversal by competing agonists.

To test the generality of p118 labeling, human U937 lymphoma cells were photoaffinity-labeled with the N4-ASA-[125I]Spd photoprobe. Prior dose-response curves comparing the U937 cells with the murine lines showed that the human lymphoma cells were sensitive to MGBG (data not shown), indicating that they expressed an analogous polyamine transport apparatus. A labeling pattern was observed similar to that seen in murine cells (Fig. 9). An intensely labeled protein appeared at ~112 kDa and may represent the human analog to murine p118. The slight molecular mass difference could be related to a different degree of glycosylation.

**DISCUSSION**

To our knowledge, this use of photoaffinity labeling demonstrates for the first time, the presence of polyamine binding proteins at the surface of mammalian cells. Previously, Morgan et al. (36) used an azidonitrobenzoyl-Spm conjugate to describe and map discrete binding sites on nucleosome core particles by photoaffinity labeling. The reagent was similar to N1-ASA-nSpm used here by having the photoprobe moiety affixed via an...
N$_1$ acyl linkage. It differed in that the probe was radiolabeled as $^{14}$C on the Spm moiety as opposed to $^{125}$I on the photoprobe moiety as used here. The present findings suggest that despite low affinity binding of polyamine receptors, photoaffinity labeling of such proteins is possible due to the irreversible bond formed during binding and photoactivation. A similar approach might also prove useful in probing small ion channels such as the N-methyl-D-aspartate receptor and inward rectifying potassium channels where polyamines have recently been shown to play a potent modulatory role (37–40).

Of the two photoprobessynthesized and used in this study, it was unexpected to find that N$_1$-ASA-nSpm was a much more effective competitor for Spd uptake than N$_4$-ASA-Spd based on our previous findings with Spd analogs (32, 33, 41). In this regard, it should be noted that although ASA is affixed to nSpm, the attachment is via an acyl linkage so that the charge on the derivatized N$_1$ is lost. Thus, with only three of the four nitrogens charged, the recognizable polyamine ligand is that of nSpd. Consistent with its ability to compete more effectively for uptake, N$_1$-ASA-nSpm labeled p118 more intensely, as indicated by the shorter exposure times required for gel autoradiography.

Of the various L1210 cell proteins cross-linked with the polyamine-conjugated photoprobos, p118 was most intensely labeled even though by Coomassie Blue staining, it represented a relatively minor membrane protein species. An analogous protein 112 kDa in size was also found on human leukemia cells. Characterization of p118 as a plasma membrane protein was based on both experimental design and findings. Labeling of intact cells at 4 °C minimizes probe internalization (42) and thus favors interaction with cell surface proteins. This bias is further enhanced by the preparation and study of plasma membrane preparations from labeled cells. Differences in p118 mobility from cells treated with neuraminidase strongly suggest that p118 contains sialic acid residues and is therefore of plasma membrane origin. Persistence of labeling following neuraminidase treatment also indicates that negatively charged sialic acid residues are probably not involved in binding photoprobos.

The specificity of p118 as a polyamine binding protein is strongly suggested by (a) similar labeling of p118 with two structurally different polyamine conjugates, (b) the absence of similar protein labeling with the unconjugated photoprobe NHS-ASA, and (c) the ability of exogenous polyamines to effectively compete in labeling of p118. The high concentration of polyamines or the analog, DESpm, required to compete with photoprobe labeling is consistent with the irreversible nature of photoprobe binding in the presence of uv light. Competitors...
This deduction was confirmed in a very recent study by Leroy et al. (35), who used a polyamine photoconjugate to map the polyamine activating site the β-subunit of casein kinase 2. In this study, competition experiments required similarly high levels of polyamines to those reported here to achieve labeling of 50%. Those authors offer a similar interpretation of their findings.

The evidence that p118 may be part of the polyamine transport apparatus is somewhat more tenuous. Comparison of plasma membrane protein labeling patterns for parental L1210 cells with polyamine transport-deficient L1210/MG cells failed to indicate a loss of p118 protein or a decrease in its labeling. Likewise, no differences were observed in any other labeled or unlabeled protein distinguishable by autoradiography or protein staining, respectively. It is possible, however, that transport deficiency is due to a functional change in the transporter protein complex (12–14, 43) and/or one of its regulatory proteins. In this regard, it is interesting that on the basis of gel mobility, p118 from L1210/MG cells consistently displayed a slightly higher molecular weight than parental line protein. This may be due to changes in the protein structure and/or to differences in post-translational modification of p118. Since the shift in p118 mobility persisted following neuraminidase treatment, post-translational differences involving sialic acid residues were excluded.

If not directly involved in polyamine transport, p118 may play a collateral role such as the folate-binding proteins play in folate transport (44–46) or function as a regulatory protein. Finally, the possibility that the polyamine binding function of p118 may be completely unrelated to polyamine transport must be considered. Polyamines, for example, have been shown to interact with the N-methyl-D-aspartate receptor (37) and inward rectifying potassium channels (38–40), both of which are on the plasma membrane. It is expected that the nature of this interesting protein will become more apparent as current purification and characterization studies progress.

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