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Differential Regulation by Retinoic Acid and Calcium of Transglutaminases in Cultured Neoplastic and Normal Human Keratinocytes

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ABSTRACT

In five lines of cultured human squamous carcinoma cells, transglutaminase activity and envelope competence were highly sensitive to retinoic acid and calcium levels in the growth medium. In cells grown in low calcium medium, these measures of keratinocyte differentiation were reduced. Retinoic acid suppressed envelope competence but total transglutaminase activity was markedly reduced, slightly affected, or greatly stimulated depending upon the cell line and whether the cells were grown in low calcium or 1.8 mM calcium-containing medium. Examination by anion exchange chromatography of the transglutaminase activity in SCC-12B2 cultures showed that expression of the particulate form (type I) of the enzyme was greatly stimulated by calcium. The increase in this activity to high levels that occurs at confluence could be almost completely suppressed by retinoic acid in the medium. The soluble form (type II) in the SCC-12B2 cell was induced in growing or confluent cultures by retinoic acid independent of the calcium concentration in the medium, but the 50% effective concentration (100 mM) for its stimulation was approximately 50-fold higher than the 50% effective concentration for suppression of the type I enzyme (2 mM). Thus, these enzymes appear to be distinct and independently regulated. This conclusion is supported by the finding that SCC-4 and SCC-9 almost exclusively expressed types II and I forms, respectively. In contrast to the results with neoplastic cells, in cultured normal epidermal cells type I enzyme comprised the overwhelming majority of activity and was only partially (75-90%) suppressible by retinoic acid, while type II enzyme seemed poorly if at all stimulable. Thus, the SCC lines appear appropriate for studying biochemical mechanisms of action of certain physiological agents, the molecular basis for altered regulation of differentiated function in neoplastic cells, and the origin of diversity within tumors.

INTRODUCTION

The formation of cross-linked envelopes, protein structures stabilized by e-(γ-glutamyl)lysine isopeptide bonding, is a characteristic feature of keratinocyte differentiation (1). This crosslinking is catalyzed by cellular transglutaminase activity. Normal human epidermal cells in vivo and in culture express a particulate (membrane bound) transglutaminase that is absent from dermal fibroblasts (2). In cultured mouse epidermal and human SCC-13 keratinocytes expression of this enzyme is stimulated by calcium in the medium and suppressed by retinoids (3–6). Normal epidermal cells also synthesize a soluble (cytosolic) transglutaminase (2, 4) that appears to correspond to the “tissue” form observed in a variety of cell types (7). Stimulation of this form by retinoids has been well documented in mouse macrophages and human HL-60 cells (8, 9). While activity of the particulate enzyme correlates well with envelope forming ability under certain culture conditions in SCC-13 cells (6), the soluble enzyme activity is unnecessary for this purpose (2) and indeed has as yet no identified function.

Carcinoma cells are often useful culture models for examining processes that are difficult to study using the normal epithelial cells due, e.g., to poor growth or loss of specialized function. A concern that can arise is to what degree the behavior observed is characteristic of normal functions. Indeed, human squamous carcinoma cells exhibit striking differences from the normal epidermal cell in culture in the regulation of envelope formation that occurs spontaneously or upon artificial transglutaminase activation using a calcium ionophore (10, 11). The present work explores the regulation of transglutaminase in 5 lines of neoplastic keratinocytes by physiological agents (retinoic acid, calcium) to provide some insight into the role of the enzyme in the aberrant expression of envelope forming ability these cells display (11). That the observed regulation has abnormal features need not detract from use of such cells to study the action of agents to which they are sensitive. Indeed, identifying cell lines with certain convenient properties could easily provide better models for that purpose than normal epidermal cells. Moreover, the generation of diversity in tumors, presumably critical for the progression to cancer, is poorly understood and may be reflected in the biochemical differences between tumor lines that can be measured in cell culture. Thus, information gained by studying biochemical variation in keratinocyte lines derived from neoplasms may advance our understanding of normal and pathological regulation of differentiated function.

MATERIALS AND METHODS

Cell Culture. Neoplastic keratinocyte lines derived from human squamous carcinomas of the tongue (SCC-4 and -9) and facial epidermis (SCC-12F2, -12B2, and -13) were cultivated with 3T3 feeder layer support (12). The keratinocytes (10⁵–10⁶/60- or 100-mm plastic culture dish) were inoculated in Dulbecco-Vogt Eagle’s medium supplemented with hydrocortisone (0.4 µg/ml) and 5% fetal bovine serum previously depleted of vitamin A by organic solvent extraction (13). After approximately 20 h, the cells were rinsed twice in PBS² and given fresh medium (changed at 4-day intervals) which was supplemented with hydrocortisone (0.4 µg/ml), vitamin A-depleted fetal bovine serum (2%), all-trans- retinoic acid (0 or 3.3 µM), and dimethyl sulfoxide (0.1%) solvent for the retinoid. For some experiments, the Dulbecco-Vogt Eagle’s medium was prepared without addition of CaCl₂, resulting in a final calcium concentration of 0.015–0.030 mM (according to atomic absorption spectrophotometry) after supplementation with serum that had been treated with Chelex-100 (Bio-Rad Laboratories) ion-exchange resin (14) in addition to solvent extraction.

Normal human epidermal cells (strain B) were cultivated with 3T3 feeder layer support in Dulbecco-Vogt Eagle’s medium supplemented with hydrocortisone (0.4 µg/ml), epidermal growth factor (10 ng/ml), cholera toxin (9 ng/ml), untreated fetal bovine serum (5%), insulin (5 µg/ml), transferrin (5 µg/ml), and triiodothyronine (20 µM). When the cultures were approximately half confluent, they were rinsed twice in PBS and switched to medium supplemented with vitamin A-depleted fetal bovine serum (10%) under conditions of interest.

Envelope Competence. The cells were disaggregated with trypsin and

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3 The abbreviation used is: PBS (137 mM NaCl-3 mM KCl-8 mM Na₂HPO₄-2 mM KH₂PO₄, pH 7.2).

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EDTA and suspended at 8 x 10^6 cells/ml in serum-free medium containing the ionophore X537A at a concentration of 50 μg/ml (Hoffman-LaRoche, Inc., Nutley, NJ). The samples were incubated for 2 h at 37°C, treated with sodium dodecyl sulfate (1%) and diithiothreitol (20 mM) at room temperature for at least 10 min and scored for envelopes by phase-contrast microscopy (15). Envelope competence was defined as the percentage of ionophore-treated cells that formed cornified envelope structures. Small errors in the total cell number (due to adherence of cells to glass surfaces) or in intact envelopes (due to possible fragmentation) may occasionally result in competence values greater than 100%. However, the relative values obtained within a given cell line were satisfactory for present purposes.

Preparation of Soluble and Particulate Extracts. Cultures held at confluence for one week except as noted were rinsed 3 times with PBS, scraped into 1.0 ml of 2 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5)-2 mM EDTA-1 mM diithiothreitol and frozen at -70°C. The samples were then thawed, disrupted with 30-40 strokes of a Dounce homogenizer and centrifuged at 150,000 x g for 30 min at 4°C. The supernatant was recentrifuged and the remaining supernatant (the soluble extract) was adjusted to 0.1% Emulgen 911, briefly sonicated, and then centrifuged at 150,000 x g for 30 min to yield the Emulgen-solubilized particulate extract.

Transglutaminase Assay. For total cell activity, cultures grown in 60-mm dishes were washed 3 times with warm PBS, scraped into 200 μl of ice-cold buffer containing 10 mM-Tris-HCl-1 mM EDTA-1% Emulgen 911 (pH 7.4) and stored frozen until assay. Twenty-five μl aliquots (0.065-0.60 mg of protein) were assayed in 0.26-ml final vol containing 0.5 mg of dimethyl casein (16), 100 mM Tris-HCl (pH 8.2), 4 mM CaCl₂, 0-0.4 mM EDTA, 5 mM diithiothreitol, 15 μM putrescine, and 0.5 μCi of [3H]putrescine (New England Nuclear). After a 30-min incubation at 37°C, protein-bound radioactivity was recovered by precipitation on glass fiber filters, rinsed with trichloroacetic acid, and measured by scintillation counting. For activities in soluble or particulate extracts, cultures grown in 100-mm dishes were prepared as described above and 100-μl (0.026-0.277 mg of protein) aliquots were assayed. Total enzyme activities calculated per unit culture surface area of cells for the fractionated SCC-12B2 cells were within 40% of those measured in unfractionated cell sonicates.

Anion-Exchange Chromatography. Soluble or particulate extracts (1-5 mg of protein) were applied to 4-cm columns of Whatman DE-52 resin equilibrated at 4°C with 50 mM Tris (pH 7.5)-1 mM EDTA-0.1% Emulgen 911-0.02% NaN₃ buffer and eluted with a linear gradient of NaCl (0-0.5 M) in this buffer. Aliquots (100 μl) of each fraction were assayed for transglutaminase activity.

Protein concentration was measured by a bicinchoninic acid (Pierce Chemical Co., Rockford, IL) modification (17) of the method of Lowry et al. (18).

RESULTS

Survey of SCC Lines. In 5 neoplastic keratinocyte lines derived from human squamous carcinomas of the face and tongue, differentiated character was found to be markedly influenced by omission of calcium from or addition of retinoic acid to the medium. From the values measured at confluence and given in Table 1 of envelope competence, transglutaminase activity, and cell density, several features of the cell responses are evident. As shown in Table 1A, in 4 of the lines (SCC-9,12B2, 12F2, and -13), a large majority of the cells (typically, 70-100%) were competent at the ordinary calcium concentration (1.8 mM) in the medium. Addition of retinoic acid at 3.3 μM during growth of the cells resulted in a nearly complete suppression of competence. A 50-85% suppression of competence was also measured when the cells were grown in low-calcium medium. Under the latter condition, addition of retinoic acid to the growth medium also resulted in the virtual elimination of envelope competence. As noted previously (11), SCC-4 has only a low ability to form envelopes under optimal culture conditions, attributable at least in part to a low content of the transglutaminase substrate involucrin (19). In the present experiments, the envelopes of the SCC-4 cells appeared more delicate and less easily discernible under phase optics than those formed by the other SCC lines (which in turn appeared less substantial than those formed by keratinocytes cultured from normal epidermis). Nevertheless, it was clear that the structures formed by SCC-4 were also greatly suppressed by retinoic acid and were even more sensitive to calcium in the medium than in the other SCC lines.

As shown in Table 1B, expression of total cellular transglutaminase activity was also markedly affected by the medium additions, but rather differently among the keratinocyte lines surveyed. In 4 of the lines (SCC-9,12B2, 12F2, and -13), activity was highest in the presence of 1.8 mM calcium (without added retinoid) and in low-calcium medium was considerably reduced, from 64 (SCC-12F2)-95% (SCC-9,-13) lower. In the presence of 1.8 mM calcium, retinoic acid suppressed enzyme expression, but to distinctly different degrees, from less than 20 (SCC-12B2) to over 93% (SCC-9). In low-calcium medium, the retinoid suppressed the enzyme in 2 lines (SCC-9 and -12F2) but had no apparent effect or stimulated it in 2 others (SCC-12B2 and -13). In striking contrast, SCC-4 expressed rather low enzyme levels except in the presence of retinoic acid, where the activity in either 1.8 mM or low-calcium medium was comparable to the maximal levels seen in the other cell lines. Since it was previously found that SCC-13 cells display little differentiated character prior to confluence, competence and transglutaminase activity in the present experiments were measured typically 1 wk after the cultures reached confluence, by which time maximal values were obtained (10). Cell density was monitored (Table 1C) since it was also a function of the medium additives. It was apparent that under a given condition of retinoic acid addition or omission, the cell density was lower in low-calcium than in 1.8 mM calcium-containing medium. Thus, in SCC-12B2 cultures grown without added retinoic acid, the cell densities differed by approximately 20%, but the difference

| Table 1 Effect of calcium and retinoic acid on envelope competence, transglutaminase activity, and cell number |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | Competence (%)  | Cell line       |                |                |                |
|                | A. Competence (%) |                | B. Transglutaminase (nmol putrescine/mg/h) | C. Cell number (in millions)/dish |
|                |                |                |                |                |                |
| Calcium level  |                |                |                |                |                |
| 0.025 (−)      | 70,85           | SCC-4           | 90,60           | 1.8, 1.3       |
| 0.18 (+)       | 100,93          | SCC-9           | 80,17           | 3.3, 3.8       |
| 0.4        | 110,102         | SCC-12B2        | 70,75           | 3.5, 3.0       |
| 0.6       | 120,126         | SCC-12F2        | 75,66           | 3.4, 3.2       |
| 1.0       | 130,131         | SCC-13          | 80,80           | 3.6, 3.4       |

- All measurements were made on cells grown on 60-mm culture dishes and held at confluence for 1 wk prior to analysis. Calcium concentrations in the medium were 0.025 (−) or 1.8 mM (+) and retinoic acid concentrations were 0 (−) or 3.3 μM (+) as indicated. A and C give values for duplicate determinations; B gives mean ± SD for triplicate determinations.
in competence (4-fold) was considerably more dramatic. Regardless of calcium concentration, the density of SCC-4,-9, and -13 cells was lower in cultures grown in the presence of retinoic acid. SCC-12B2 cells showed this effect only in low-calcium medium. In contrast, the density of SCC-12F2 cells appeared higher in retinoic acid-containing medium, but the measurements probably reflected loss of cells by desquamation from the cultures held at confluence without retinoid. Overall, the various small effects of growth conditions on cell density had no obvious correlation with either competence or transglutaminase activity.

Differential Regulation in SCC-12B2. Although previous results showed that envelope competence was closely correlated with transglutaminase activity in SCC-13 in medium with 1.8 mM calcium (6), the present survey results were incompatible with any such simple correlation for all the SCC cell lines. However, the contrast in stimulation or inhibition of activity in SCC-4 and -9 suggested that keratinocyte lines could express 2 different forms of transglutaminase that were regulated in opposite directions by retinoids. As a stringent test of this hypothesis, the enzymes in SCC-12B2 cells were examined in detail since retinoic acid had little net effect on total activity despite almost complete inhibition of competence.

Homogenates of SCC-12B2 cultures, grown in medium containing 1.8 mM calcium with or without retinoic acid, were separated into soluble and particulate extracts and chromatographed on DEAE-cellulose columns in the presence of Emulgen 911 nonionic detergent. As shown in Fig. 1, the cells expressed activities eluting at approximately 0.1 (type I) and 0.2 M NaCl (type II). The type II form was greatly stimulated by retinoic acid (Fig. 1A), whereas the type I form was almost entirely suppressed (Fig. 1, B and C), in parallel with envelope competence. Over 90% of type II activity was associated with the soluble cell fraction. These experiments also indicated that the particulate activity in cultures just reaching confluence was noticeably lower (Fig. 2B) than the level attained in cultures held for 1 wk at confluence (Fig. 1C). In such chromatographs, activity eluting at 0.1 M NaCl was found only in the particulate fraction, unlike cultures of normal and SCC-13 cells, and occasionally as a doublet peak as illustrated; some evidence for chromatographic heterogeneity of the particulate enzyme has also been obtained using SP-Sephadex (2).

Fig. 2 shows the effect of retinoic acid on expression of transglutaminase in cells grown in low-calcium medium. Since the 2 enzyme forms were cleanly separable by solubility, column chromatography proved unnecessary and the 2 forms were assayed in the presence of nonionic detergent in the supernatant and pellet fractions obtained by high-speed centrifugation. The soluble (type II) activity was clearly stimulated as in cells grown in 1.8 mM calcium. Suppression of the particulate (type I) enzyme was not obvious, however, since this form was virtually absent in low-calcium medium. Thus, the expression of these 2 forms of transglutaminase differed dramatically in dependence upon calcium in the medium.

In addition to suppression of type I versus stimulation of type II, differences in the regulation of transglutaminase by retinoids were also conspicuous in 2 other types of experiments. Fig. 3 shows the activity of the particulate and soluble enzymes as a function of retinoic acid concentration. From the results illustrated, it was apparent that the concentration of retinoic acid suppressing type I by 50% (2 nM) was 50-fold lower than that required to give half-maximal stimulation of type II (100 nM).

Differential regulation of transglutaminase expression was also evident upon examination of rapidly growing versus confluent cultures. Fig. 4 shows the effects of retinoic acid on the 2 enzymes as measured in cultures that were half confluent, newly confluent, and confluent for 1 wk. As illustrated in Fig. 4A, the soluble enzyme (type II) was highly stimulated (over 4-fold) at each time point measured. In contrast, the low level of particulate enzyme activity (Fig. 4B) seen in preconfluent cells was not detectably affected, whereas the 5- to 10-fold increase ordinarily occurring after confluence was suppressed.

Contrasts in Other Lines. The hypothesis that SCC-4 and -9 synthesize predominantly enzymes of types II and I, respectively, was tested by column chromatography. Indeed, as seen in Fig. 5, virtually all the activity expressed by SCC-9, found in the particulate fraction of cell homogenates, eluted in the position of the type I enzyme (Fig. 5B). This activity was highly sensitive to retinoid suppression, as illustrated. A trace of activity eluting in the position of the type II enzyme was detected only in the soluble fraction of cells grown in retinoic acid (Fig. 5A). SCC-9 thus appears even more deficient in expression of type II activity than does SCC-13, previously
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Fig. 2. Effect of growth in low (0.025 mM)- or high (1.8 mM)-calcium medium on expression of soluble and particulate transglutaminase activity in SCC-12B2 cells. Cultures treated with (■) or without (○) 3.3 μM retinoic acid were held at confluence for 1 wk prior to extraction. Bars, range of values of duplicate cultures.

Fig. 3. Effect of retinoic acid concentration on soluble and particulate transglutaminase activities in SCC-12B2 cells grown in 1.8 mM calcium-containing medium. Cultures were held at confluence for 1 wk prior to extraction to yield particulate (■) and soluble (○) fractions. ---, approximate concentrations giving half-suppression of particulate activity and half-stimulation of soluble activity; bars, range of values of duplicate cultures (if not visible, range was less than the size of the point).

Fig. 4. Transglutaminase activities in SCC-12B2 cultures grown to different densities in 1.8 mM calcium-containing medium in the presence (■) or absence (○) of 3.3 μM retinoic acid. Prior to extraction, remaining 3T3 feeder cells were removed by spraying the cultures with PBS containing 0.5 mM EDTA. Bars, range of values of duplicate cultures.

Fig. 5. Anion exchange chromatography of soluble (A) and particulate (B) transglutaminase activities of SCC-9 cells grown in 1.8 mM calcium-containing medium in the absence (■) or presence (○) of 3.3 μM retinoic acid. I, type I; II, type II.

Due to retinoid toxicity in these experiments, the agent was added when the keratinocyte colonies were well established. The soluble and particulate extracts of the cells, held for 1 wk at confluence, were examined by ion-exchange column chromatography (Fig. 7). The results showed that the normal cells expressed very high levels of type I activity that were only partially inhibited by retinoid. In 3 experiments, retinoic acid inhibited particulate transglutaminase activity by 75–90%, still yielding a level of activity comparable to the untreated highly competent SCC-12B2 line. Consistent with this finding, and as previously reported in human conjunctival keratinocytes (20),

noted to lack this form in the absence of added retinoid (6) but in which retinoic acid can be seen to stimulate total cellular activity in the absence of calcium (Table 1B). In marked contrast to the SCC-9 cells, SCC-4 expressed considerable retinoic acid stimulatable activity in the soluble fraction and only very little retinoic acid inhibitable activity in the pellet (Fig. 6). This property provides a further rationale for the relatively low ability of SCC-4 cells to form ionophore-induced envelopes (Table 1).

In contrast to the sensitivity of all the SCC lines tested, transglutaminase activity in cells cultured from normal epidermis was not as highly responsive to retinoic acid in the medium.
experiments but in each case was stimulated only marginally if at all (Fig. 7, top).

In one experiment, cells grown in low-calcium medium with retinoic acid were 60% competent, while cells grown in parallel in 1.8 mM calcium but also in the presence of exogenous retinoic acid were 106% competent, again suggesting only limited sensitivity. Type II enzyme was detected in most Chromatographie acid were 106% competent, again suggesting only limited sen

paral diabetic acid were 60% competent, while cells grown in parallel in 1.5 HIMcalcium-containing medium in the absence (•) or presence (O) of 3.3 MM retinoic acid. //, type II.

Fig. 7. Anion exchange chromatography of soluble (A) and particulate (B) transglutaminase activities from normal human epidermal cells (strain B) grown in 1.5 mm calcium-containing medium in the absence (O) or presence (C) or 3.3 μM retinoic acid. I, type I; II, type II.

DISCUSSION

The present study of transglutaminase and its regulation in 5 neoplastic human keratinocyte lines has revealed several important phenomena. To begin with, considerable variation was evident among the lines in the extent of expression of particulate (type I) and soluble (type II) transglutaminase. The measured values of envelope competence and total cellular enzyme activity (Table 1) are readily reconcilable, since the capacity to form envelopes is closely correlated with the level of particulate transglutaminase in the various SCC lines under different culture conditions. That the type II enzyme can function effectively to stabilize envelopes seems implausible but cannot be ruled out. In that case, retinoic acid could reduce competence by suppressing participating substrate proteins, a number of which have been identified (21). In SCC-13, for example, involucrin is suppressed 75–80% by retinyl acetate (10). Regulation of competence simply by insertion of type II enzyme into the membrane to yield the type I form (22) appears untenable in view of our evidence that the 2 enzymes are under different regulatory control. In any event, our data highlight the value of the envelope competence measurement as reflecting an intermediate stage of differentiation that is highly affected by the physiological conditions of the culture.

It is well known that tumors of a given cell type show great variation in properties and that within a given tumor heterogeneity is also apparent (23). Reflecting such variation in culture, the SCC lines studied here are known to differ significantly from each other in a number of properties including growth or survival in semisolid medium (11), growth requirements (12), and keratin polypeptides (24). The present study of transglutaminase expression supports at a biochemical level this phenomenon of variation even in the SCC-12B2 and -12F2 lines, sibling clones derived from the same skin lesion (19).

Despite the dramatic quantitative differences among the SCC lines, they all showed a striking sensitivity to retinoic acid compared to normal epidermal cells. Enhanced sensitivity to retinoids has been observed previously in SCC-13 with respect to envelope competence (10, 25) and type I transglutaminase (6) and in SCC-12 and -15 with respect to keratin synthesis (26). These findings all indicate that the process of neoplastic change characteristically elicits in the afflicted keratinocytes a state of enhanced sensitivity to physiological agents. That the sensitivity extends beyond retinoids is suggested by the response of most of the SCC lines to calcium deprivation (Table 1) and the response of SCC-13 to corticosteroids (6). Such a sensitivity itself could be a source of altered differentiation that neoplastic cells display in vivo.

From this perspective, it may prove useful to imagine the existence of an altered physiological state in which neoplastic cells in different early lesions exhibit certain common properties and from which they generally diverge with time. Good evidence for such a process has been obtained in preneoplastic nodules of the rat liver (27). Thus, in epidermis and related epithelia, determining the state(s) at which enhanced retinoid sensitivity arises could prove useful not only for understanding the dynamics of neoplastic progression but also perhaps for diagnostic purposes. In the latter respect, transglutaminase provides a potentially useful marker to complement the keratins (28–30), involucrin (31), and other keratinocyte differentiation markers. From a practical standpoint, moreover, the SCC lines offer valuable models in which to study mechanisms of retinoid action as well as relative efficacy of therapeutic derivatives in human keratinocytes.
While the present work was in progress it was reported that mouse epidermal keratinocytes in primary culture express particular and soluble transglutaminases that respond to retinooids and calcium in essentially the same fashion as we have shown for human SCC lines (4). This important finding resolves the apparent paradoxical relation between stimulation of transglutaminase activity and suppression of spontaneous envelope formation by retinoic acid in these cells (32). Interestingly, the mouse cultures are highly responsive to the induction of the soluble tissue transglutaminase when grown in low-calcium medium but are said to respond poorly if pretreated with elevated calcium concentration. In the latter state, they resemble normal human epidermal cells, the sensitivity of which is not affected much by calcium deprivation. Overall, the present work reveals considerable similarity between mouse and human transglutaminase regulation that was not apparent previously.

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