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Internalization of *E. coli* ST mediated by guanylyl cyclase C in T84 human colon carcinoma cells

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Abstract

Internalization of *Escherichia coli* heat-stable enterotoxin (ST) mediated by guanylyl cyclase C was examined in T84 human colon carcinoma cells. Surface-associated, receptor-bound ST was quantitatively separated from intracellular ligand employing acidic guanidine-HCl. ST was internalized in a time-, temperature-, and ligand concentration-dependent fashion only by cells specifically expressing guanylyl cyclase C. Only receptors which bound reversibly to ST appeared to mediate endocytosis. The rate of internalization of ST empirically determined in these studies was 0.23 min^{-1}. The density of surface receptors for ST was similar at 4°C and 37°C, suggesting that these receptors recycle back to the cell surface following internalization of ligand. Similarly, internalized ST was rapidly cleared from the intracellular compartment following endocytosis. These studies demonstrate that ST undergoes ligand-dependent receptor-mediated endocytosis in human colon carcinoma cells.

Keywords: Guanylyl cyclase C; *E. coli* heat-stable enterotoxin; Internalization; T84 human colon carcinoma cell; ST receptor

1. Introduction

Enterotoxigenic *Escherichia coli*, an etiologic agent responsible for infectious diarrhea in developing countries, induces fluid and electrolyte secretion in the intestine by elaborating a low molecular weight heat-stable toxin, ST [1–5]. ST produced by *E. coli* is an 18 or 19 amino acid peptide that induces intestinal secretion and diarrhea upon binding to specific receptors, GCC, localized in the brush border of intestinal enterocytes [6–8]. These receptors are members of a family possessing ligand binding and guanylyl cyclase catalytic domains on a single transmembrane protein [8]. ST–receptor interaction leads to activation of guanylyl cyclase and increases in intracellular cGMP [6–8]. This cyclic nucleotide directly mediates fluid and electrolyte transport by activating a protein kinase and altering phosphorylation of, and chloride flux through, the cystic fibrosis transmembrane conductance regulator [9,10].

Endocytosis of ligand–receptor complexes is a common property of receptors which contributes to metabolism of the ligand, regulation of cell surface receptor density, and termination of the signaling cascade [11,12]. However, little is known concerning the role of endocytosis in the regulation of signaling mediated by receptor guanylyl cyclases. Natriuretic peptides are similar to ST in that they are low molecular weight heat-stable peptides which regulate cardiovascular homeostasis by interacting with specific guanylyl cyclase-associated receptors in various target tissues (GCA and GCB; [8]). Interestingly, there is heterogeneity in the ability of these different, but related, receptors to undergo receptor-mediated endocytosis. Thus, GCA receptors do not undergo internalization upon binding of ANP in cultured renal mesangial or renomedullary interstitial cells [13]. In contrast, GCB receptors are internalized upon binding of ligand and these receptors are actively recycled back to the cell surface following intracellular processing [14]. To date, the role of receptor-mediated endocytosis in transmembrane signaling induced by ST and the fate of this toxin bound to cell surface receptors remains undefined. The present study examines

Abbreviations: ANPs, atrial natriuretic peptides; CFTR, cystic fibrosis transmembrane regulator; DMEM, Dulbecco's minimal essential medium; cGMP, guanosine 3′,5′-cyclic monophosphate; GCA, guanylyl cyclase A (ANP receptor); GCB, guanylyl cyclase B (C-type natriuretic peptide receptor); GCC, guanylyl cyclase C (heat-stable enterotoxin receptor); ST, heat-stable enterotoxin; PBS, phosphate-buffered saline

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whether GCC undergoes internalization and the characteristics of that process in T84 cells derived from a human colonic adenocarcinoma metastatic to lung [15].

2. Materials and methods

2.1. Materials

T84 and COS-7 cells were obtained from the American Type Culture Company (Rockville, MD). ST was a generous gift of Dr. D. Robertson, Department of Biochemistry and Microbiology, University of Idaho, Moscow, Idaho. Tissue culture supplies were obtained from Gibco laboratories (Grand Island, NY). All other chemicals were of the highest analytical grade and obtained from Sigma (St. Louis, MO).

2.2. Cell culture

T84 and COS-7 cells were cultured in 1:1 Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) containing 5% (v/v) fetal bovine serum supplemented with penicillin and streptomycin at 37°C in an atmosphere of 5% CO_2/95% O_2 [16]. Media were replaced every 2–3 days. Cells were subcultured every 6–7 days. Cells were allowed to grow to confluence prior to subculturing into 24-well plates. After subculturing, confluent wells were incubated for an additional 24 h at 37°C prior to use in experiments. Studies were conducted with 10^6 cells/well as determined by manual counting using a hemocytometer. There was less than 10% variability in cell number/well.

2.3. Iodination of ST

ST was iodinated to a specific activity of 1000–2000 Ci/mmol and purified as described previously [16]. [^{125}I]ST possesses full efficacy and potency in assays of receptor binding and guanylyl cyclase activity [16].

2.4. Dissociation of cell surface-bound [^{125}I]ST

Confluent T84 cells in 24-well plates were incubated with 10 nM [^{125}I]ST in 200 μl of buffer containing DMEM/Ham's F12, 0.1% bovine serum albumin, pH 7.4 (binding media) at 4°C for 3 h. After incubation, binding media was aspirated and cells washed three times with ice-cold PBS (500 μl/wash) followed by addition of 500 μl of ice-cold dissociating buffer and incubation for 10 min at 4°C. In some experiments, cells with [^{125}I]ST bound to surface receptors were incubated with binding buffer containing 0.025% trypsin for 15 min at 4°C [17]. After incubation, dissociating buffer was aspirated and cells were washed with an additional 500 μl of the same buffer. The two aliquots of dissociating buffer were combined and radioactivity quantified in a Packard gamma counter. To quantify residual cell-associated [^{125}I]ST, remaining cells were solubilized with 500 μl of 1 N NaOH for 60 min at 37°C. Dissociating agents tested included: acidic glycine buffer (50 mM glycine/150 mM NaCl, pH 2.5) acidic glycine buffer containing 2 M urea, 0.025% trypsin, and acidic guanidine buffer (2 M guanidine/150 mM NaCl, ph 2.5).

2.5. Binding of [^{125}I]ST to T84 cells in the absence of endocytosis

Assays were performed at 4°C to quantify binding of [^{125}I]ST to intact T84 cells in the absence of receptor endocytosis or recycling [11,12,14,18]. Confluent T84 cells in 24-well plates were washed twice with 500 μl of binding media. Binding was initiated by the addition of 200 μl of binding media containing 10 nM [^{125}I]ST. At various times binding was terminated by aspirating the media and washing the cells three times with ice-cold PBS to remove residual free [^{125}I]ST. Cell surface-bound [^{125}I]ST was recovered by incubating the cells with acidic guanidine buffer to dissociate [^{125}I]ST from its receptor, as described above. Residual cell-associated radioactivity, which represents endocytosed ligand, was recovered by solubilizing cells with 1 N NaOH for 1 h at 37°C. Nonspecific binding was determined in parallel incubations in the presence of a 100-fold excess of unlabeled ST.

2.6. Internalization of [^{125}I]ST

Internalization of [^{125}I]ST by T84 or COS-7 cells was quantified in assays performed at 37°C. Confluent cells in 24-well plates were washed twice with 500 μl of 37°C binding media prior to each experiment. Cells were incubated with 200 μl of binding media containing increasing concentrations of [^{125}I]ST for various times up to 8 h, as indicated. At specific times, binding media was aspirated and cells washed three times with 500 μl of ice-cold PBS. Surface-bound and intracellular radioactivity was quantified as described above using acidic guanidine buffer and NaOH, respectively.

2.7. Miscellaneous

Protein was determined as described by Bradford (Bio-Rad, Richmond, CA). Binding isotherms for Scatchard analyses were plotted and binding constants calculated using 'Cigale' [16]. In some studies (FigsFig. 1.Fig. 2 Fig. 31, 3), double reciprocal plot analyses, in which the reciprocal of binding was plotted against the reciprocal of time, was employed to estimate equilibrium binding [19]. Linear regression analyses were performed using 'Cricket Graph' on a Macintosh IIci personal computer. In general, correlation coefficients for linear regression analyses were > 0.95. Results are representative of at least 2 experiments. Error bars represent standard error (S.E.) unless otherwise indicated.

3. Results

3.1. Dissociation of [^{125}I]ST from cell surface receptors

Previous studies demonstrated that [^{125}I]ST does not quantitatively dissociate from receptors [20–22]. Indeed, about 30% of specifically bound [^{125}I]ST appears to be irreversibly associated with binding sites in intestinal mucosal cells [21]. Since quantification of internalized ST is predicated on the ability to completely dissociate and remove cell surface-bound ligand, agents demonstrated previously to dissociate ligand–receptor complexes were examined for their ability to dissociate [^{125}I]ST from receptors on T84 cells. Of the dissociating agents tested, incubation with guanidine acidic buffer (2M guanidine/150 mM NaCl, pH 2.5) at 4°C for 10 min consistently removed greater than 95% of the surface-bound [^{125}I]ST from T84 cells (Table 1). This is in close agreement with the ability of this agent to dissociate radiolabeled ST from receptors in cell-free assays [20]. Other agents were less effective or highly variable in their ability to quantitatively strip [^{125}I]ST from surface receptors of T84 cells. Interestingly, acidic glycine buffer is an effective stripping agent which quantitatively removes natriuretic peptides from guanylyl cyclase-coupled receptors, yet this buffer removed only about 80% of the [^{125}I]ST from GCC on the surface of T84 cells [13,14]. Treatment of T84 cells with acidic guanidine buffer did not alter the integrity of the plasma membrane, since cells exposed to this buffer continued to exclude trypan blue.

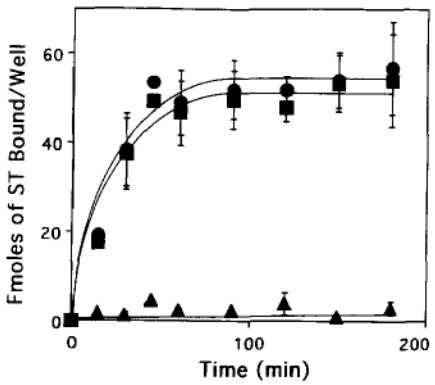

Fig. 1. Binding and internalization of [^{125}I]ST to T84 cells at 4°C. T84 cells (10^6 cells/well) were incubated with 10^{-8} M [^{125}I]ST and incubated for 2.5 h to equilibrium, as described in Section 2. Specific binding was calculated by subtracting non-specific binding, determined in parallel incubations in the presence of excess (10^{-6} M) unlabeled ST, from total binding. At equilibrium, cells containing total cell-associated radioactivity (circles) were washed with dissociation buffer to remove cell surface-bound-radioactivity (squares). The radioactivity remaining with these washed cells represents radioactivity in the intracellular compartment (internalized; triangles). Results are representative of at least three experiments. Error bars, S.E.

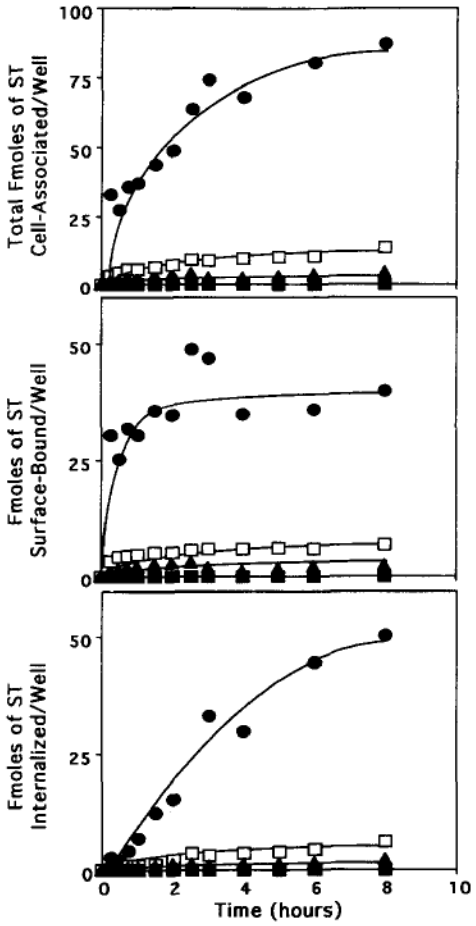

Fig. 2. Time-course of cell surface binding and internalization of increasing concentrations of [^{125}I]ST in T84 cells at 37°C. Incubations were conducted and non-specific binding was quantified as described above. Internalized ligand was separated from cell surface-bound ligand with dissociation buffer as described above. Concentrations of labeled ST employed included 0.1 nM (triangles), 1 nM (open squares), and 10 nM (circles). Upper panel, total cell-associated ligand; middle panel, cell surface-bound ligand; lower panel, internalized ligand. Closed squares represent the total cell-associated, surface-bound, and internalized radioactivity when COS-7 cells, which do not possess GCC, were incubated with 10 nM [^{125}I]ST. These studies are representative of at least three experiments.

3.2. [^{125}I]ST binding to T84 cells at 4°C

[^{125}I]ST bound to T84 cells at 4°C in a time-dependent and saturable fashion (Fig. 1). Employing 10^{-8} M ligand, binding equilibrium was achieved in about 60 min. Analysis of these data by double reciprocal plot demonstrated that maximum binding of [^{125}I]ST was 50 fmole ST/10^6 cells, using 10^{-8} M ligand. There was no internalization of [^{125}I]ST in T84 cells at 4°C, since virtually all of the radioactivity associated with cells could be dissociated using acidic guanidine buffer. These data agreed closely with earlier studies demonstrating minimal internalization of ligands by receptor-mediated endocytosis at 4°C [11,12,17]. In agreement with these observations, cell surface ST receptor density remained constant throughout the

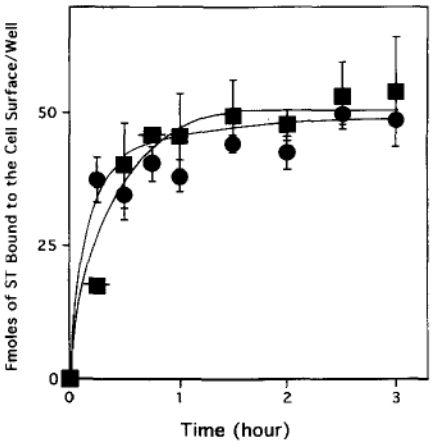

Fig. 3. Comparison of the binding of [^{125}I]ST to T84 cells at 4°C (squares) and 37°C (circles). Specific binding of radioligand to T84 cells at different temperatures was quantified as described in Section 2 and Figs. 1 and 2. Data are representative of at least three experiments. Error bars, S.E.

3 h incubation in the presence of [^{125}I]ST, once equilibrium was achieved. These observations demonstrate that internalization of [^{125}I]ST does not occur and there is no appreciable down-regulation of ST receptors at 4°C despite persistent exposure to free ligand.

3.3. Binding of [^{125}I]ST to T84 cells at 37°C

Total radioactivity reflecting the association of [^{125}I]ST with T84 cells increased in a time-and concentration-dependent fashion upon incubation of these cells with increasing concentrations of that ligand at 37°C (Fig. 2A). Association of radiolabeled ligand with T84 cells was specific since that association could be completely blocked by incubation with an excess of unlabeled ST. Furthermore, cell-associated radioactivity could not be detected when the highest concentration of [^{125}I]ST (10^{-8} M) was incubated with COS-7 cells, which do not express GCC receptors, for 8 h [23].

Total radioactivity associated with T84 cells resulting from their incubation with [^{125}I]ST at 37°C could reflect ligand bound to cell surface receptors and/or radioactivity in the intracellular compartment reflecting internalized ligand–receptor complexes. ST associated with surface receptors was quantified at various times by incubating T84 cells with acidic guanidine-HCl, as described above (Fig. 2B). ST bound to cell surface receptors in a concentration- and time-dependent and saturable fashion. Cell surface binding appeared to reach equilibrium at 37°C in a concentration-independent fashion, with equilibrium achieved by 60 min with all ligand concentrations examined (data not shown). [^{125}I]ST binding to the surface of T84 cells is specific since it can be completely competed with excess unlabeled ligand. Similarly, COS-7 cells do not exhibit measurable binding of this ligand at the highest concentrations studied (10^{-8} M).

3.4. Internalization of [^{125}I]ST at 37°C

[^{125}I]ST internalized by endocytosis was quantified at each time point by dissolving cells, whose surface receptor-associated ligand previously had been dissociated with acidic guanidine buffer, with 1.0 N NaOH, as described above (Fig. 2C). Radioactivity increased in T84 cells incubated with increasing concentrations of [^{125}I]ST at 37°C in a time-and concentration-dependent fashion. This is in contrast to results obtained at 4°C, in which radioactivity could not be detected in the intracellular compartment after dissociating ligand from surface receptors with acidic guanidine (Fig. 1). Thus, accumulation of ST in the intracellular compartment is a temperature-dependent process. Also, accumulation of ST intracellularly could be completely competed by performing these experiments in the presence of excess unlabeled ligand. These data suggest that intracellular accumulation of ST is mediated by specific receptors. In close agreement with these observations, radioactivity could not be detected in the intracellular compartment of COS-7 cells, which lack ST receptors, when these cells were incubated with 10^{-8} M [^{125}I]ST. The specificity, temperature sensitivity, and concentration- and time-dependence of the accumulation of radioactivity in T84 cells suggests that ST is internalized by a receptor-mediated process involving the specific interaction of the ligand and GCC.

3.5. Down-regulation and receptor recycling of GCC

The density of surface receptors for ST appear to be similar on T84 cells at 4°C and 37°C (compare Fig. 1 and Fig. 2B). These data suggest that the steady-state concen-

Table 1
The effect of various dissociating agents on the binding of [^{125}I]ST to T84 cells at 4°C

Treatment [a]	% [^{125}I]ST dissociated from cell surface receptors [b]
None	0
0.2 M acetic acid/0.5 M NaCl, pH 2.5	60 ± 6
50 mM glycine/0.1 mM NaCl, pH 2.5	83 ± 9
2 M guanidine-HCl/0.15 M NaCl, pH 2.5	98 ± 1
0.025% Trypsin	35 ± 1

[a] T84 cells were incubated with 10 nM [^{125}I]ST at 4°C for 2 h, to equilibrium, and subsequently incubated with the dissociating buffer as described in Section 2. Studies were conducted at 4°C to minimize endocytosis of ligand–receptor complexes.
[b] % [^{125}I]ST dissociated from cell surface receptors = (radioactivity associated with cells after incubation with dissociating agent)/(radioactivity associated with cells before incubation with dissociating agent).
[c] Dissociation of [^{125}I]ST from cells with trypsin was performed as described in Section 2.

tration of GCC at the surface of these cells is temperature- and ligand-independent. To examine this further, T84 cells were incubated with near-saturating concentrations (10 nM) of [^{125}I]ST at 4°C and 37°C for up to 3 h and specific cell surface-bound ligand was quantified at various times (Fig. 3). Binding to cell surface receptors was time-dependent and saturable at both temperatures. Equilibrium was achieved rapidly, at the earliest time point sampled (15 min), at 37°C whereas it was achieved more slowly (45 min) at 4°C, as expected. Maximum binding achieved at equilibrium at 10^{-8} M, calculated by double reciprocal plot analysis of the time course of binding of [^{125}I]ST to cell surface receptors, was similar at 37°C (50 ± 3 fmol/10^6 cells) and 4°C (55 ± 7 fmol/10^6 cells). As demonstrated above, there is no detectable internalization of [^{125}I]ST at 4°C. Therefore, receptor density quantified at this temperature represents the full complement of GCC at the cell surface, in the absence of endocytosis. The number of surface receptors for ST on T84 cells were similar at 4°C and 37°C, demonstrating that GCC does not undergo down-regulation, desensitization, or depletion from the cell surface upon persistent exposure to ligand. Similarly, that receptor density is unchanged at 4°C and 37°C, although internalization of ligand occurs at the higher temperature, suggests that GCC may be rapidly recycled back to the membrane following ligand-induced endocytosis. Rapid recycling of cell surface receptors, without down-regulation at the cell surface, following receptor endocytosis has been observed previously [11,12,17,26–32].

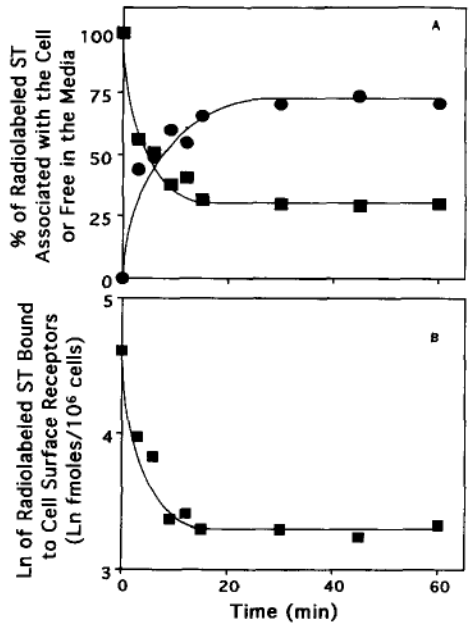

Fig. 4. Kinetics of [^{125}I]ST bound at 4°C to T84 cells which were subsequently increased to 37°C. T84 cells (10^6 cells/well) were incubated with 10 nM [^{125}I]ST for 2.5 h to equilibrium at 4°C. At the end of incubation, free radioligand was removed, cells were washed three times with ice-cold binding buffer, and washed cells were placed in binding buffer warmed to 37°C. At various times, radioactivity free in the media (circles) and bound to the surface of cells (squares) was quantified as described in Section 2 (A). The time-course of the association of radioactivity with the cell surface after transfer of cells to 37°C was analyzed on a semilogarithmic plot (B). These studies are representative of at least three experiments.

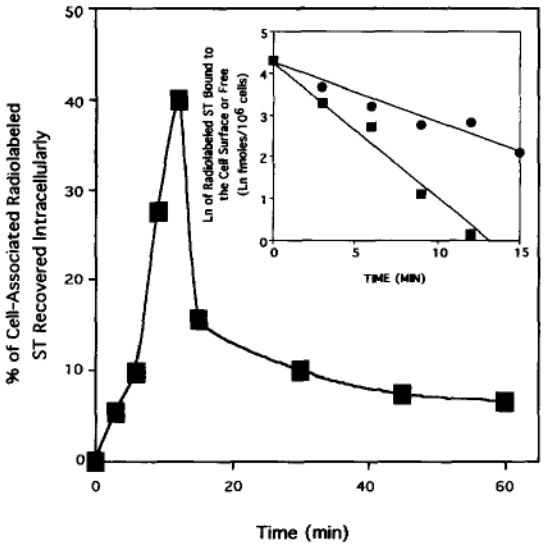

Fig. 5. Kinetics of internalization of [^{125}I]ST bound at 4°C to T84 cells which were subsequently increased to 37°C. Studies were performed as outlined in Fig. 4 and radioactivity in the intracellular compartment was quantified as described in Section 2. Inset, semilogarithmic plot of the time course of radiolabeled ST bound to the cell surface (squares) or free in the media (circles; from Fig. 4). These studies are representative of at least three experiments.

3.6. Kinetics of surface-bound [^{125}I]ST in T84 cells

The fate of [^{125}I]ST bound to surface receptors in T84 cells was directly examined. In these experiments, cell surface ST receptors were pre-bound with [^{125}I]ST by incubating T84 cells with 10 nM [^{125}I]ST at 4°C for 2.5 h. At this temperature, there is no internalization of radioligand and binding reaches equilibrium within 45 min (see Fig. 1,3). After incubation at 4°C, cells were placed at 37°C with fresh binding media which did not contain free [^{125}I]ST and the fate of cell surface-bound radioactivity was followed (FigsFig. 4.Fig. 5 4, 5).

When cells were warmed from 4°C to 37°C, there was a rapid decrease in cell-associated [^{125}I]ST within the first 9 min, to about 30% of that initially bound at 4°C (Fig. 4A). A parallel rapid increase in radioligand was observed in the binding media. The semilogarithmic plot of the loss of cell-associated radioactivity exhibits a curvilinear isotherm, suggesting multiple processes and/or receptor populations contributing to different rates of loss of ST from cells (Fig. 4B). The initial rapid rate of loss of cell-associated radioactivity most likely corresponds to both dissociation of ligand–receptor complexes at the surface and degredation or recycling and release of ST at the cell surface. The terminal (> 15 min) rate of loss of cell-associated radioactivity is very slow and occurs in the absence of internaliza-

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