A Former Amino Terminal Signal Sequence Engineered to an Internal Location Directs Translocation of Both Flanking Protein Domains

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ABSTRACT To determine whether a functional amino terminal signal sequence can be active at an internal position, a hybrid gene was constructed in which the entire coding region of bovine preprolactin cDNA was inserted into chimpanzee α-globin cDNA 109 codons downstream from the initiation codon of globin. When RNA synthesized in vitro from this plasmid (pSPGP1) was translated in the rabbit reticulocyte cell-free system, a 32-kD protein was produced that was both prolactin and globin immunoreactive. When microsomal membranes were present during translation (but not when added posttranslationally), a 26-kD and a 14-kD product were also observed. By immunoreactivity and electrophoretic mobility, the 26-kD protein was identical to mature prolactin, and the 14-kD protein appeared to be the globin domain with the prolactin signal sequence attached at its carboxy terminus. From (a) posttranslational proteolysis in the presence and absence of detergent, (b) sedimentation of vesicles in the presence and absence of sodium carbonate pH 11.5, and (c) N-linked glycosylation of the globin-immunoreactive fragment after insertion of an Asn-X-Ser N-linked glycosylation site into the globin coding region of pSPGP1, it appears that all of the 26-kD and some of the 14-kD products, but none of the 32-kD precursor, have been translocated to the lumen of the membrane vesicles. Thus, when engineered to an internal position, the prolactin signal sequence is able to translocate both flanking protein domains.

These data have implications for the understanding of translocation of proteins across the membrane of the endoplasmic reticulum.

The mechanism by which newly synthesized secretory proteins are translocated across the membrane of the endoplasmic reticulum (ER) is an unsolved problem. It is generally accepted that signal sequences play a role in this process, but it is unclear whether they serve as ligands for proteinaceous receptors in the membrane and activate a catalytic translocation mechanism (1), or whether they trigger translocation across the bilayer spontaneously, i.e., without the participation of other membrane proteins (2).

Other fundamental aspects of the translocation process are equally obscure: Is the energy to drive these events derived from the thermodynamics of protein–lipid interactions, from protein synthesis, or does proteinaceous (enzymatic) machinery in the membrane actively move the protein across the bilayer? To what extent is signal sequence function constrained by the nature of the chain being translocated and the location of the signal sequence within that chain? What role, if any, does signal sequence cleavage play in the process of translocation? Why are secretory proteins completely translocated across the bilayer, whereas integral membrane proteins only partially translocated?

Our laboratory is attempting to address such issues in protein translocation by engineering defined coding regions into various and unusual locations or orientations and using these constructions as expression probes in cell-free systems that are amenable to fractionation and reconstitution. As a first step toward understanding signal sequence function we previously demonstrated that a signal sequence coding region...
FIGURE 1 Structure of pSPGP1 fusion plasmid. (A) Construction scheme for pSPGP1, in which the entire coding region of bovine preprolactin was inserted into an SP6 plasmid containing chimpanzee δ-globin cDNA such that the signal sequence of the resulting hybrid fusion protein is located 109 amino acids from the amino terminus (see Materials and Methods for details). Chimpanzee δ-globin coding regions are indicated by white bars and preprolactin by stippled bars. The black bars indicate the SP6 promoter, and arrows on the plasmid diagrams indicate the direction of transcription. (B) Restriction map of the preGSP coding region of pSPGP1 with corresponding gene product represented underneath. The white bar represents the 109 amino acid globin domain (G) and the stippled bar the 229 amino acids of preprolactin (P). The 30 amino acid signal sequence of prolactin is also indicated (S). The upward pointing arrowhead indicates the transcription start site. (C) Restriction endonuclease analysis of pSPBP3, pSPGP1, and pSPGI E. Plasmid DNA (5 μg) was digested in a volume of 10 μl with NcoI and SphI. Samples were prepared and electrophoresed on a 5% polyacrylamide gel in Tris-Borate-EDTA. Lane 1, pSPBP3; lane 2, pSPGP1; lane 3, pSPGI E; lane 4, pBR322 digested with HindIII as size markers in kilobases.
engineered at the amino terminus was sufficient to translocate the normally cytoplasmic protein globin across microsomal membranes in cell-free systems (3). Similarly, we demonstrated the functional distinction of signal and stop transfer sequences, and that stop transfer sequences require preceding signal sequences for expression of transmembrane integration by the ability or inability of these sequences, engineered into various coding regions, to convert nascent globin domains into secreted versus integral transmembrane proteins (4).

To determine whether a previously amino terminal signal sequence can function from an internal position in a protein, we have engineered the signal sequence of bovine preprolactin between the coding regions for globin and prolactin, and then engineered an N-linked glycosylation site into the globin coding region. Here we present the surprising behavior of these molecules in cell-free systems and their implications for a variety of issues in protein translocation.

MATERIALS AND METHODS

Materials: All restriction endonucleases, nuclease Bal 31, calf intestinal alkaline phosphatase, SP6 RNA polymerase, T4 DNA ligase, and Klenow fragment of E. coli DNA polymerase were from Boehringer Mannheim Diagnostics, Inc., Houston, TX or from New England Biolabs, Beverly, MA. RNase inhibitor was from Promega Biotec, Madison, WI; staphylococcal protein A-Sepharose was from Pharmacia, Inc., Piscataway, NJ; rabbit anti-human globin serum was from Cappel Laboratories, Cochranville, PA; rabbit anti-ovine prolactin was from United States Biochemical Corp., Cleveland, OH; proteinase K was obtained from Merck, FRG; endoglycosidase H (Endo H) and [3H]methionine (translation grade, >800 Ci/mmol) were from New England Nuclear, Boston, MA; Nikkol (octa-ethylene glycol mono-n-dodecylether), a non-ionic detergent) was from Nikko Chemicals Co., Ltd., Tokyo, Japan. Plasmid pSPBP3 was constructed by William Hansen, Department of Biochemistry and Biophysics, University of California at San Francisco, using bovine preprolactin cDNA (18, 28). All globin-encoding plasmids were derived from pMC18 (4).

Construction of Globin-Prolactin Fusion Plasmid, pSPGPI: As depicted in Fig. 1, plasmid pSPBP3, containing the entire coding region for bovine preprolactin, was linearized with NcoI in the presence of ethidium bromide and the overhang filled in by treatment with E. coli DNA polymerase I Klenow fragment in the presence of all four dNTPs. The plasmid was then cut with PstI and the 850-base pair (bp) fragment containing the preprolactin coding region was purified, and eluted from a 1% low melting point agarose gel. Plasmid pSPG1E was cut with BamH1, the 5' overhang filled in as described above, then cut with PstI and the 3-kilobase (kb) vector gel purified. The purified pSPG3 fragment and pSPG1E vector were treated with T4 DNA ligase. After transformation of E. coli, plasmid DNA was prepared from individual ampicillin-resistant colonies and screened by restriction enzyme analysis with HindIII. The 430-bp HindIII fragment of the positive clone, pSPG3, was inserted into pSPGP1 which had been digested to completion with HindIII and treated with calf intestinal alkaline phosphatase to prevent self-ligation. E. coli were transformed and DNA prepared from individual ampicillin-resistant colonies were screened with NcoI to determine the presence and correct orientation of the HindIII insert.

Rabbit Reticulocyte Lysate Transcription-coupled Translation: SP6 plasmids were transcribed in vitro (5) at a concentration of 0.2 mg/ml in a reaction mix containing 40 mM Tris-HCl pH 7.5, 6 mM MgCl2, 2 mM spermidine, 10 mM dithiothreitol, 25 μg/ml calf liver RNA, 0.5 mM each ATP, CTP, and UTP, 0.1 mM GTP, 0.5 mM GpppG, 0.9 U/μl RNase inhibitor, and 0.4 U/μl SP6 RNA polymerase. Reactions were carried out at 40°C for 1 h and aliquots used directly in transcription-linked translations in the rabbit reticulocyte lysate cell-free system at a concentration of 20%. Translation reactions were carried out in 20-200 μl vol that contained 43% rabbit reticulocyte lysate (prepared as described, reference 6), 100 mM KCl, 2 mM MgCl2, 0.9 mM GTP, 1 mM ATP, 10 mM creatine phosphate, 0.2 mM each of 19 amino acids minus methionine, 16 mM Tris HCl (pH 7.5), 0.44 mM spermidine, 2 mM dithiothreitol, 0.4 mg/ml creatine phosphokinase, 0.1 mg/ml calf liver RNA, and 1 μCi/ml [3H]methionine. Reaction mixtures were incubated at 24°C for 60 min.

Protein Processing and Translation Assays: In vitro transcripts of SP6 plasmids were translated separately in a rabbit reticulocyte cell-free system in the presence or absence of intact dog pancreas rough microsomes (prepared as described, reference 7). Translation products were immunoprecipitated and separated by SDS PAGE. Bands were localized by autoradiography and quantitated by densitometer scanning of preflashed film, using an LKD.
Membrane-dependent Processing of Nascent Pre GSP to GSI and P1

Upon expression in a transcription-coupled rabbit reticulocyte lysate cell-free translation system, pSPGP1 encoded a fusion protein of ~32 kDa with both globin and prolactin immunoreactivity (Fig. 2, lanes A-D) called pGSP. When translation reactions were supplemented with microsomal membranes two additional translation products, not present in the absence of membranes, were seen after electrophoresis on polyacrylamide gels in SDS with subsequent autoradiography (Fig. 2, lane G). One of these bands, termed P1, was found to be anti–prolactin but not anti–globin immunoreactive and to co-migrate with authentic mature bovine prolactin (Fig. 2, lane F). The other product, termed GSI, was anti–globin but not anti–prolactin immunoreactive, and migrated with an apparent molecular weight slightly greater than that of authentic full-length globin (14 kDa, Fig. 2 lane E). When membranes were added after completion of protein synthesis with further incubation, neither of these bands were generated (data not shown).

The difference in the relative intensities of the bands in the autoradiographs is due to the methionine distribution in the [35S]methionine-labeled, newly synthesized proteins. Upon processing, the eleven methionines in pre GSP, P1 contains seven and GSI only four.

Fig. 3 demonstrates that when membranes were present during translation, GSI and P1 were generated in a 1:1 ratio and that the percentage processing of both products increased correspondingly with membrane concentration. These data indicate that GSI and P1 are generated from a common

RESULTS

Construction of Globin–Prolactin Fusion Plasmid

Fig. 1, A and B shows the scheme by which the entire coding region for bovine preprolactin was inserted, in frame, 109 codons downstream from the initiation codon of a chimpanzee α-globin cDNA clone previously engineered behind the SP6 promoter (pSPG1E). Putative positive colonies were selected for ampicillin resistance and screened for the presence of restriction fragments of predicted sizes of the globin, signal sequence, and prolactin coding domains, as well as the SP6 promoter. These characteristic restriction fragments from both parent plasmids, pSPG1E and pSPBP3, and from the new construction, pSPGP1, are displayed in Fig. 1 C.
(nascent) precursor and that the processing activity is associated with the microsomes.

Our interpretation of these results is that the prolactin signal sequence, now localized internally, is still functional as evidenced by accessibility of the signal sequence cleavage site to signal peptidase, a luminaly disposed enzyme of the endoplasmic reticulum (9). The products of this cleavage are authentic prolactin (P1) and globin with the prolactin signal sequence attached at its carboxy terminus (GS1).

Both GS1 and P1 are Localized inside Microsomal Vesicles

To determine which of these products are completely translocated across the membranes, we used two different experimental approaches. First, posttranslational proteolysis with proteinase K was used to localize the precursor and the two cleavage products. Any polypeptide that is completely translocated across the bilayer will be resistant to proteolysis unless the integrity of the membrane is abolished by the addition of nonionic detergents (7). If a protein spans the membrane, its molecular weight will be reduced by digestion of the cytoplasmically disposed domains (10, 11). A protein localized completely outside of the membrane vesicles will be totally digested by the added protease (3, 4). As can be seen in Fig. 4, the precursor, preGSP, is completely degraded (lanes H–J). However, some chains of GS1 (23%) and almost all chains of P1 (89%) were protected from proteolysis (bands were quantitated by scanning densitometry as described in Materials and Methods; data not shown). When nonionic detergent was included with the protease to disrupt the protecting lipid bilayer, all protection from proteolysis was abolished (lanes J and L). To rule out the possibility that the relatively poor protection of GS1 was due to a slightly increased intrinsic protease resistance of that molecule relative to preGSP, rather than to translocation across a protecting lipid bilayer, we investigated the kinetics of proteolysis. We varied both the time of protease digestion (from 15 to 90 min) and the protease concentration (from 0.1 to 0.4 mg/ml final concentration of proteinase K). At all time points and at all protease concentrations virtually none of the preGSP chains but the same percentage of GS1 chains were protected (<1% and ~20%, respectively, as determined by scanning densitometry; data not shown). Our interpretation of these data is that the now internal prolactin signal sequence is able to direct not only the subsequently synthesized prolactin domain across the microsomal membrane, but also the already completed globin domain, albeit with lower efficiency.

**Engineering an N-Linked Glycosylation Site into the Globin-coding Region of pSPG1**

To further test our interpretation we engineered a glycosylation site into the globin-coding region of pSPG1. Previously...
we have shown that artificial glycosylation sites engineered into either the BssHII site (~20 amino acids from the amino terminus) or the BstE II site (~110 amino acids from the amino terminus) of globin result in translation-coupled core glycosylation of the globin domain when an amino terminal signal sequence fuses precisely to the amino terminus of chimpanzee globin coding regions of pSPSG3 (upper drawing) and pSPgGP1 (lower drawing). The stippled bar represents prolactin, and white bars represent globin domains. The arrowhead pointing upward indicates the position of the Asn-X-Ser glycosylation site in the globin molecule encoded by pSPSG3; the branched symbol represents the carbohydrate that is added to the Asn of the Asn-X-Ser inserted into pSPgGP1 (but is not added to the translation product of pSPSG3; see Fig. 7).

Our interpretation of these data was confirmed by treatment with Endo H which shifted the position of GS1' on SDS PAGE to that of GS1 (Fig. 7, lanes D–F), thereby demonstrating the presence of carbohydrate on GS1'. Similar treatment of the protein encoded by pSPSG3, which contains the glycosylation site but lacks a signal sequence, demonstrates neither shift up on SDS PAGE with co-translational membranes (Fig. 7, lane B) nor protection from proteolysis (data not shown) nor shift down with Endo H digestion (Fig. 7, lane C).

**Carbonate Extraction of gGS1**

Having demonstrated unequivocally that both P1 and G1' were faithfully translocated across microsomal membranes using the internal signal sequence of prolactin encoded in pSPgGP1, we proceeded to study the disposition of these molecules in the lumen. In particular, we wanted to know whether it was soluble in the lumen, i.e., did G1' display properties of a secretory or of an integral membrane protein?

**Glycosylation and Protection of gGS1**

Fig. 6 demonstrates that when pSPgGP1 is transcribed and translated as described previously for pSPGP1, a ~32-kD globin and prolactin immunoreactive protein was synthesized (lanes D and F, arrows pointing downward). The co-translational (but not posttranslational, see lane C) addition of microsomal membranes resulted in appearance not only of the 26-kD P1 band (lane J) and the 14-kD GS1' band (lane E, small arrowhead pointing upward), analogous to GS1 in the presence of pSPGP1, but also in the appearance of a 16-kD globin but not prolactin immunoreactive band (lane E, large arrow pointing upward). This new 16-kD band, termed G1', was believed to be the glycosylated derivative of GS1. Consistent with this interpretation, GS1' was well protected from proteases (lane F), as was P1 (lane K), while GS1 was relatively poorly protected (lane F, small arrow pointing upward). Protection of GS1' approximated that of GS1 (20%), presumably representing those chains of GS1 that were translocated but not glycosylated, an intermediate often observed in glycoprotein biosynthesis both in vivo and in vitro (Lingappa, V., unpublished observation). Also, as was seen with preGSP, the precursor, pre GS1, was not protected.
Similarly we investigated whether pre gGSP and those gGS1 chains that are not protected from proteases (Fig. 6, lane F) are peripherally bound to membranes, integrated into membranes, or free in the cytosol. Fig. 8 shows the results of sedimentation of membranes in either isotonic sucrose buffer or after extraction with sodium carbonate pH 11.5, a procedure designed to strip off nonintegral proteins and content proteins from microsomal membranes (8). It can be seen that all forms (pre gGSP, gGS1, gGS1', and P1) are extracted by carbonate (lanes E–H) and that both P1 and gGS1' sedimented quantitatively with membranes in sucrose buffer (lanes A–D). Control extractions with a translation product known to integrate into membranes co-translationally demonstrated the fidelity of carbonate extraction in our hands (data not shown). Both gGS1 and pre gGSP were found in both the supernate and the pellet after membrane sedimentation in sucrose. The majority of gGS1 sedimented with microsomes, while pre gGSP was split approximately evenly between the membrane pellet and the supernate.

These data indicate that gGS1' is not integrated into the vesicle membrane but exists either free in the microsomal lumen or peripherally associated with membrane proteins of the vesicle lumen. Also, those chains of gGS1 that are not protected from proteolysis are apparently in large measure bound to the membranes. The significance of this finding is currently under investigation.

DISCUSSION

Our original purpose in constructing pSPGPI was to determine if amino terminal signal sequences were functionally related to internal signal sequences by converting a normally amino terminal signal into an internal one.

The existence of internal signal sequences has been proposed (1, 14) for integral membrane proteins as a means of accounting for multiple transmembrane loops, since alternating signal and stop transfer sequences in register could serve to stitch the nascent polypeptide into the bilayer in a programmed fashion. Recent evidence demonstrates the existence of internal signal sequences directly in the case of vertebrate rhodopsin (Friedlander, M., and G. Blobel, personal communication; Lingappa, V. R., manuscript in preparation) and hepatitis B virus surface antigen (Eble, B., V. R. Lingappa, and D. Ganem, manuscript submitted for publication) by the ability of internal coding regions to translocate domains that would otherwise remain in the cytoplasm. The existence of internal signal sequences in secretory proteins is more controversial. Ovalbumin lacks a cleaved signal sequence (15), yet its nascent chain competes with nascent preprolactin for membrane receptors for translocation (16), suggesting the existence of an uncleaved signal sequence somewhere in the ovalbumin chain. An internal tryptic fragment from ovalbumin displayed rough ER-specific competition for membrane receptors involved in prolactin translocation. Because this internal region, but not the amino terminus, displayed strong homology to amino terminal signal sequences of other proteins of the chicken oviduct, it was proposed that the uncleaved signal sequence of ovalbumin resided in this internal position rather than at the amino terminus and therefore, that amino terminal and internal signal sequences were structurally and functionally similar (14). Subsequent work, both indirect (17, 18) and direct (19), has demonstrated the existence of a signal sequence for ovalbumin that is closer to, but not necessarily at, the amino terminus, but has not ruled out the existence of a signal sequence at the more internal location.

In principle, internal and amino terminal signal sequences could represent two classes of ligands. For example, amino terminal signal sequences might be capable of directing the translocation only of polypeptide synthesized subsequently while internal signal sequences could translocate both preceding and subsequently synthesized domains. Our demon-
FIGURE 7  Endo H digestion of products encoded by pSPSG3 and pSPgGP1. Plasmid pSPSG3 (lanes A–C) and pSPgGP1 (lanes D–F) were transcribed and translated in separate reactions in the presence (+ mb., lanes B, C, E, and F) or absence (− mb., lanes A and D) of dog pancreas membranes (4 A280 U/ml). Samples were immunoprecipitated with globin antiserum and digested with endoglycosidase H (Endo H +, lanes C and F) or mock digested (Endo H −, lanes B and E) as described in Materials and Methods. The small arrow pointing downward in lane E indicates gGSI', apparently the glycosylated form of gGS1 (large arrows pointing upward, lanes E and F). Large arrows pointing downward indicate pre gGSP1 (lanes D–F), and small arrows pointing upward indicate translation products of pSPSG3.

The finding that the two cleavage products are translocated with different efficiencies while using a common signal sequence is surprising. It suggests that GS1 and P1 domains of nascent preGSP are translocated independently. Perhaps this reflects a difference in translocation between domains that are nascent (such as P1 which is synthesized subsequent to the signal sequence) versus those that have already folded (such as the globin domain that is synthesized before the signal sequence). The location of the signal sequence relative to the translocated domain (i.e., at the carboxy terminus of globin versus at the amino terminus of prolactin) may also affect translocation efficiency of one versus the other domain, as might retention or removal of the signal sequence. Finally, we cannot rule out the possibility that the signal sequence retained by the globin domain can direct the reverse reaction of transport of GS1 from lumen to cytosol, to some extent, hence giving the appearance of inefficient translocation into the lumen. It should be possible to determine the kinetics of GS1 and P1 translocation as well as to evaluate the various explanations for the different efficiencies of translocation, using this cell-free expression system.

Translocation of the globin domain of 109 amino acids that is synthesized before the signal sequence is a remarkable observation with surprising implications for the mechanism of chain translocation. Two models for translocation of nascent polypeptides across the ER membrane have been put forth (1, 2). One of these postulates a proteinaceous transport tunnel whose assembly in the plane of the lipid bilayer is directed by the signal sequence (1). In this view, both targeting and translocation are the result of ligand–receptor-like inter-
actions between domains of nascent chains (topogenic sequences) and membrane proteins serving as receptors and transporters; the other model predicts that the free energy gain from insertion of the hydrophobic signal sequence directly into the lipid bilayer as a helical hairpin more than offsets the unfavorable energetics of pulling the contiguous hydrophilic chain into the bilayer, and thereby provides a thermodynamic basis for spontaneous insertion and translocation across the membrane, with the signal sequence retained in the bilayer after cleavage. This model requires no proteins in the membrane to facilitate translocation, and can be modified to account for signal recognition particle and its receptor as a requirement for targeting rather than translocation.

Since protein folding is likely begun during the window of time between initiation of protein synthesis and extrusion of the signal sequence from the large subunit of the ribosome (20, 21), it appears that a (partially) folded globin domain is presented to the membrane for translocation during the synthesis of nascent preGSP. Any hypothesis for translocation must account not only for this observation but also for the apparently soluble, nonintegrated form in which the gGS1’ chains are found. The topogenic sequence model can comfortably account for both of these considerations by postulating either an enzyme capable of denaturing the folded globin domain before transport as a component of the translocation machinery, or that the proteinaceous tunnel is of a dimension large enough to accomodate a partially folded domain. While the spontaneous insertion model might accomodate the idea of translocation of a folded globin domain providing it is denatured before the process of translocation, the observation that the gGS1’ chains are not integrated into the microsomal membrane argues that the signal sequence was never inserted directly into the bilayer.

Thus, regardless of whether a partially folded or completely denatured domain is translocated, our results suggest that not only targeting (i.e., signal recognition particle–receptor interaction [22–24]) but also translocation itself, involves proteinaceous machinery in the membrane.

Another implication of the globin domain being synthesized before the signal sequence, is that the energy expended in synthesis of a length of polypeptide cannot be the sole driving force for its translocation. We cannot at this time rule out a role for continued synthesis of the prolactin domain in translocation of the already completed globin domain, although we doubt this possibility. It seems more likely to us that the driving force for translocation resides in the membrane. If this hypothesis is correct, it should be possible to dissociate translocation of arrested nascent domains from continued protein synthesis, although the inability of completed and released chains to be translocated argues for some additional requirement such as for the ribosome or other protein cofactors (such as SRP) in the cytoplasm.

The placement of a globin domain at the amino terminus of a signal sequence provides the first marker for localization of a signal sequence after its cleavage. While we cannot rule out that such a bulky group has altered the fate of the signal, these data suggest that the signal is itself translocated, although it may be pulled back into the cytosol for degradation subsequently. In any case it appears not to be retained in the lipid bilayer, in view of our results with carbonate extraction.

We have also demonstrated a novel and direct approach to assaying for transfer of proteins across the ER membrane, that is the engineering of an N-linked glycosylation site (Asn-X-Ser/Thr) into the protein domain of interest. While failure to glycosylate does not rule out translocation, the addition of carbohydrate can be a powerful independent line of evidence in studying these events.

These results change our understanding of the vectorial discharge of proteins synthesized on membrane-bound ribosomes (25). Since in this case, translocation of the globin domain must take place after completion of its synthesis, it would appear that only a subset of the early events culminating in segregation in the ER cisternae need be vectorial.

Finally, the translocation of a globin domain when placed amino terminal to a signal sequence should not be taken to imply that any protein domain regardless of size or of secondary structure would be so translocated. The limits on translocation of domains both amino terminal as well as carboxy terminal to a signal sequence remain to be determined. There may well exist domains or conformations incompatible with translocation. At least two cases of cytoplasmic proteins unable to be translocated by amino terminal signal sequences have been proposed (26, 27). Further studies in cell-free systems should resolve these issues.

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