Expression and potential functions of G-protein α subunits in embryos of Xenopus laevis

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Summary

During early embryonic development, many inductive interactions between tissues depend on signal transduction processes. We began to test the possibility that G-proteins participate in the signal transduction pathways that mediate neural induction. The expression during Xenopus development of three Go subunits, Goα, Gα1, and Gαs-1, was characterized. The three maternally expressed genes showed different expression patterns during early development. Whole-mount in situ hybridization revealed that all three genes were expressed almost exclusively in the gastrula ectoderm and predominantly in the neuroectoderm in the neurula embryo. In order to investigate the involvement of these proteins in neural induction, we overexpressed the G-protein α subunits by injecting the Gα mRNAs into fertilized eggs. Overexpression of Gαs-1 increased the ability of gastrula ectoderm to become induced to neural tissue approximately four-fold. Overexpression of Goα and Gα1,1 had less pronounced effects on neural competence, and inhibition of the Goα and Gα1,1 proteins by pertussis toxin did not change the neural competence of the exposed gastrula ectoderm. Overexpression of the Goα and Gα1,1 genes did, however, inhibit the normal disappearance of the blastocoel during gastrulation, suggesting a role for these G-proteins in regulating this process. The data also suggest a specific role for the Gαs subunit in mediating the initial phases of neural induction.

Key words: Xenopus laevis, signal transduction, G protein, neural induction.

Introduction

Neural induction is an intercellular interaction which takes place during gastrulation when dorsal mesoderm induces the overlying dorsal ectoderm to form neural tissue. Recently, we have shown that the protein kinase C (PKC) and cAMP signal transduction pathways are involved in mediating neural induction (Otte et al., 1988, 1989, 1990, 1991; Otte and Moon, 1992). For many growth factors and hormones it has been shown that the activation of G proteins is an important intermediate step in the activation of the PKC and cAMP signal transduction cascades. Upon ligand-receptor interaction, G-proteins are activated, and this leads in turn to the activation of effector molecules like phospholipase C or adenylyl cyclase. The heterotrimeric G-proteins are composed of three subunits, α, β and γ. The α subunits are thought to be primarily responsible for the specificity by which the G-proteins are coupled to specific effector molecules, although recent evidence also indicates that the β and γ subunits of certain G-proteins are able to activate specific effector molecules (Federman et al., 1992).

Given the extensive participation of signal transduction processes in early development, it would be surprising if G-proteins did not play important roles in embryogenesis. However, only recently have studies reported the existence of G-proteins in the early development of higher organisms. Complex changes in the temporal and spatial expression patterns of Gα subunits occur during mouse (Jones et al., 1991) and Drosophila development (Wolfgang et al., 1991). Using antibodies against the Gα protein, it was shown that Gα protein was expressed in the embryos of the amphibian Pleurodeles walti (Pituello et al., 1991). Both pertussis toxin-sensitive and -insensitive G-proteins are present in the Xenopus laevis egg and fertilization is a pertussis toxin-insensitive event (Kline et al., 1991). Important additional clues about the functions of G-proteins in early development come from genetic studies of Drosophila. One gene, concertina, encodes a Gα-like protein and is involved in gastrulation (Parks and Wieschaus, 1991). Another gene, sevenless, encodes a tyrosine kinase that plays a pivotal role in the development of the Drosophila eye (Rubin, 1991), and ras-like, G-protein-like factors are an intrinsic part of the sevenless signal transduction pathway (Simon et al., 1991; Bonfini et al., 1992).
In this paper, we started to investigate the possibility that G-proteins participate in the signal transduction pathways that mediate neural induction. The availability of *Xenopus* cDNAs encoding the Go, Ga₁ and Ga₅ subunits (Olate et al., 1989, 1990) allowed us to characterize the developmental timing of expression and spatial localization of these Gα transcripts. To investigate the potential involvement of these Gα subunits in neural induction or other developmental processes, we manipulated the expression of the subunits by injecting the Gα mRNAs into fertilized eggs. Our data suggest, in particular, a role for the Ga₅ subunit in mediating the initial phases of neural induction and suggest a role for the Go and Ga₅ proteins in the development of the blastocoeel.

**Materials and methods**

**RNA injection and analysis**

Capped synthetic RNA was made by in vitro transcription and microinjected into fertilized eggs as described (Moon and Christian, 1989). RNA was extracted and RNAase protection assays were performed (Moon and Christian, 1989; Otte and Moon, 1992). Levels of Gαo, Ga₁ and Ga₅ transcripts were determined by synthesizing antisense riboprobes in the presence of [α-32P]UTP (Amersham), then hybridizing the probes to RNA blots. Whole-mount in situ hybridization was performed according to Harland (1991).

**In vitro ADP-ribosylation of Gαo and Ga₅ subunits using pertussis toxin**

ADP-ribosylation was performed as described (Kline et al., 1991; Kopf and Woolkalis, 1991). Samples containing ADP-ribosylated Gα subunits were either analyzed directly on an SDS-10% polyacrylamide gel, or subjected to immunoprecipitation, using polyclonal antibodies against Gαo or Ga₅ (Goldsmith et al., 1988a, b). The mammalian peptides against which the antibodies were raised were completely conserved in *Xenopus* (Olate et al., 1989, 1990). Immunoprecipitation was carried out in 250 μl immunoprecipitation buffer consisting of 20 mM Tris pH 7.5; 0.1% SDS; 1% NaCl; 0.1% deoxycholate; 1% Triton; 0.5 mM EDTA and 0.25% β-mercaptoethanol and 20 μg/ml of either Gαo or Ga₅ antibody. Samples were rotated at 4°C for 16 hours, 3 mg protein A Sepharose was added and rotated for an additional 3 hours. The immunoprecipitate was washed five times with 500 μl buffer before analysis on an SDS-10% polyacrylamide gel.

**Results**

**Developmental timing and localization of expression of the Xenopus Gαo, Ga₁ and Ga₅ genes**

As a necessary prelude to investigating the possible functions of *Xenopus* Gαo, Ga₁ and Ga₅ subunits, we first examined the temporal expression of these genes through early embryonic development as well as the spatial localization of the transcripts. When total RNA from several developmental stages was analyzed by northern blot analysis, we detected an approximately 4 and 7 kb transcript for the Gαo gene, a single approximately 4.5 kb transcript for Ga₅ and a single transcript of approximately 4 kb for Ga₅ (Fig. 1). All transcripts were expressed maternally.

**Fig. 1.** Expression of Gαo, Ga₁ and Gα₅ transcripts through early *Xenopus* development. (A) Antisense probes for the *Xenopus* Gαo, Ga₁ and Gα₅ genes (Olate et al., 1989) were used for northern analysis of total RNA (20 μg) isolated from the indicated developmental stages (Nieuwkoop and Faber, 1967). The filter was reprobed with a probe for 5S ribosomal RNA.

The highest expression level of the 4 kb Gαo transcript was found in the fertilized egg throughout gastrula stages (Fig. 1) and the expression level decreased during neurula stages. The level of expression of the 7 kb transcript, on the other hand, was low during the earliest developmental stages and increased during the neurula stages (Fig. 1). The expression of the Ga₅ transcript was highest in the fertilized egg throughout gastrula stages and declined during neurula stages. No major changes were observed in the expression level of the Ga₅ transcript from the fertilized egg throughout neurula stages.

The timing of expression of these three *Xenopus laevis* Gα subunit genes differs to some extent from the recently reported timing of expression and localization of the Gαo gene in another amphibian, *Pleurodeles waltl* (Pituello et al., 1991). In that species, expression of the Gαo protein starts at the time that the ectoderm becomes competent to be induced to form neural tissue. We found that, in *Xenopus*, the Gαo as well as the Ga₁ and Ga₅ genes were
Fig. 2. Localization of Gαo, Gαi-1 and Gαs transcripts in early gastrula and neurula embryos. Whole-mount in situ hybridization was performed on stage-10 early gastrula (A, B, C) and stage-14 early neurula (D, E, F) albino embryos. In gastrula embryos, the dorsal (D) and the ventral side (V) side of the embryo are indicated. The anterior (ANT) to posterior (POST) orientation of the neurula embryos, the position of the archenteron (ARCH) and the neural plate (N) are indicated. The stained embryos were subsequently sectioned (G, H, I, early gastrula and J through O, early neurula). The blastocoel (BL) of gastrula embryos (G, H, I), the neuroectoderm (N), the underlying dorsal mesoderm (M) (J, K, L) and the ventral ectoderm (VE) (M, N, O) are indicated. Bar shown in G, 100 μm for each panel.
Fig. 4. Effects of overexpressing $G_\alpha_0$, $G_\alpha_1$, and $G_\alpha_4$ mRNA on the development of the blastocoel. (A) Fertilized eggs were injected with 100 pg $G_\alpha_0$, $G_\alpha_1$, or $G_\alpha_4$ mRNA and cultured to neurula stage. Embryos injected with $G_\alpha_0$ mRNA developed normally with the neural plate facing upwards. The embryos injected with $G_\alpha_0$ or $G_\alpha_1$ mRNA turned upside down, resulting in the ventral side facing upwards. (B) The residual blastocoel of an embryo that was injected with $G_\alpha_1$ mRNA was punctured and the embryo was turned, to show that the neural plate (N) developed normally. (C) The blastocoel of embryos that were injected with $G_\alpha_0$ or $G_\alpha_1$ mRNA remained as a blister on the ventral side of neurula embryos up to stage 20. (D) A normal stage-15 embryo was fixed and cut in half to show the development of the archenteron (ARCH) and disappearance of the blastocoel (BL). (E) A stage-15 embryo that had been injected with $G_\alpha_1$ mRNA was fixed and cut in half to show the retention of the blastocoel (BL). Note the absence of a normally developed archenteron. The endoderm is pushed against the dorsal mesoderm, underlying the neural plate.
expressed throughout early development. Also the G\(\alpha_6\) and G\(\alpha_8\) proteins were expressed throughout early development, as determined by whole-mount immunocytochemistry (data not shown), using polyclonal antibodies against conserved peptide sequences of the G\(\alpha_6\) or G\(\alpha_{6-1+2}\) subunits (Goldsmith et al., 1988 a, b).

We next examined the spatial localization of the G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) transcripts in the early stage-10 gastrula and early stage-14 neurula embryos. Stage 10 marks the beginning of neural induction (Nieuwkoop and Faber, 1967). Whole-mount in situ hybridization of gastrula embryos revealed that all three genes were expressed almost exclusively in the gastrula ectoderm (Fig. 2A, B, C), both in the inner and outer cell-layer of the ectoderm (Fig. 2G, H, I). Subtle differences were revealed in that the transcripts of the G\(\alpha_6\) gene were found to be localized more towards the vegetal hemisphere as compared to the transcripts of the other two genes (Fig. 2A, B, C). Importantly, we did not observe a major difference in distribution between dorsal and ventral ectoderm. In neurula embryos, all three genes were expressed predominantly in the neuroectoderm (Fig. 2D, E, F and J, K, L), but a low level of expression was found in the ventral ectoderm as well (Fig. 2M, N, O). Importantly, all three genes were expressed in the neuroectoderm with no expression in the underlying mesoderm or presumptive notochord, as revealed when the whole-mount stained embryos were embedded in paraffin and sectioned (Fig. 2J, K, L). Whole mount immunocytochemistry, using the polyclonal antibodies against G\(\alpha_6\) and G\(\alpha_{6-1+2}\) (Goldsmith et al., 1988 a, b) revealed that the localization of the G\(\alpha_6\) and G\(\alpha_{6-1}\) proteins in the early gastrula and neurula embryo was similar to the localization of the transcripts of these genes (data not shown).

In summary, northern blot analysis of the expression of the three G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) subunits reveals a differential timing of expression in development and whole-mount in situ hybridization shows strong localized expression of all three genes in the ectoderm of early gastrula embryos and predominant expression in the neuroectoderm of early neurula embryos.

*Changed G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) expression affects the neural competence of gastrula ectoderm to a different extent*

The above observation that G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) transcripts are present in the ectoderm of the stage-10 early gastrula when neural induction starts (Nieuwkoop and Faber, 1967), and are present in the neural induced ectoderm of early neurula embryos, is consistent with the possibility that G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) have a role in neural induction. The localized expression of the transcripts in these developmental stages also raises the possibility that manipulating the levels of G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) mRNA may affect neural competence. We directly tested this possibility by injecting a range of 5 to 500 pg G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) mRNA into the fertilized egg. The mRNAs translated well in an in vitro translation assay (data not shown). At gastrula stage the whole ectoderm was dissected, divided in two equal pieces and recombined with equal sized pieces of stage-10 dorsal marginal zone mesoderm (Otte et al., 1991; Otte and Moon, 1992). Dorsal mesoderm from normal embryos was taken in order to avoid interference of overexpression of G\(\alpha_6\), G\(\alpha_{6-1}\) and G\(\alpha_{6-2}\) with the neural inducing capacity of the dorsal mesoderm. The recombinates were cultured to stage-22 tailbud, total RNA was extracted and the expression of XIF3, an intermediate filament gene which is an anterior neural marker (Sharpe et al., 1989), and N-CAM, which is a general neural marker (Kintner and Melton, 1987), was analyzed by employing an RNase protection assay. Denistyom of the autoradiographs of six independent experiments revealed that injection of G\(\alpha_6\) and G\(\alpha_{6-1}\) mRNA led to an approximately two-fold increase of both XIF3 and N-CAM expression in both dorsal (Fig. 3A; G\(\alpha_6\), lane 3; G\(\alpha_{6-1}\), lane 5) and ventral (G\(\alpha_6\), lane 4; G\(\alpha_{6-1}\), lane 6) recombinates, compared to control dorsal (lane 1) and ventral (lane 2) recombinates. In five independent experiments, a greater increase, of approximately four-fold, was observed in levels of both XIF3 and N-CAM transcripts following overexpression of G\(\alpha_{6-1}\) (dorsal, lane 7 and ventral, lane 8).

The existing difference between control dorsal and ventral recombinates (lanes 1 and 2) in their ability to become neural induced (Sharpe et al., 1987; Otte et al., 1991; Otte and Moon, 1992) remained following overexpression of each of the three G\(\alpha\) subunits. This indicates that these G\(\alpha\) subunits are not involved in the mechanisms that underly the dorsal-ventral differences in neural competence.

The injected mRNA of all three G-proteins was still present during gastrula stages (result not shown). Given that overexpressing the G\(\alpha_6\) and G\(\alpha_8\) genes neither increased nor decreased neural competence to a great extent, we tested whether the amounts of G\(\alpha_6\) and G\(\alpha_8\) proteins were elevated. In order to do this, we used the ability of pertussis toxin to ADP-ribosylate and hence radioactively label the G\(\alpha_6\) and G\(\alpha_8\) proteins. The ADP-ribosylated gastrula G\(\alpha_6\) and G\(\alpha_8\) proteins were subsequently immunoprecipitated using the polyclonal antibodies against conserved peptide sequences of the respective subunits (Goldsmith et al., 1988a, b). As shown in Fig. 3B, both the antibodies against the G\(\alpha_6\) protein (lanes 1 and 2) and the G\(\alpha_8\) protein (lanes 3 and 4) immunoprecipitated an approximately 40 ×10^3 M\(_f\) protein. A 39 and 40 ×10^3 M\(_f\) protein has been predicted, based on the sequence of the G\(\alpha_6\) and G\(\alpha_8\) cDNAs, respectively (Olate et al., 1989, 1990). Injection of both G\(\alpha_6\) (lane 2) and G\(\alpha_8\) (lane 4) mRNA led to an increase of the respective ribosylated protein as compared to control levels (lanes 1 and 3).

Another approach to study the possible involvement of the G\(\alpha_6\) and G\(\alpha_8\) proteins in neural induction is to expose the responding tissue to pertussis toxin, thereby inhibiting the activity of the G\(\alpha_6\) and G\(\alpha_8\) proteins, and to examine subsequently the neural competence of the exposed ectoderm. In order to expose the ectoderm as long as possible, the pertussis toxin was injected into the blastocoe1 of stage-7 blastula embryos. These embryos were cultured to stage-10 early gastrula before the ectoderm was excised and recombined with dorsal mesoderm from normal embryos. The inner layers of the ectoderm had therefore been exposed to pertussis toxin for more than 5 hours. No effects were observed on the levels of XIF3 and N-CAM expression in the cultured recombinates, indicating that neural induction had not been influenced by pertussis toxinin.
Presence of Goo or pg mRNA embryonic (lanes 3 and 4) or maternal (lanes 1 and 2) Gcri-1 mRNA, and Gcri-r protein (lanes 3 and 4) was induced by 70-80% decrease in ADP-ribosylation. The observation that pertussis toxin is unable to inhibit or increase neural induction suggests that neural induction is mediated through pertussis toxin-insensitive signal transduction pathways, which is consistent with the limited effects of overexpressing the pertussis toxin-insensitive Gcri-1 and Gcri-r proteins.

Phenotypical effects of overexpressing Gcri-1 and Gcri-r

In order to explore further a possible function of the Gcri-1 and Gcri-r proteins, the embryos that were injected with Gcri-1 mRNA subunit mRNAs were cultured beyond gastrulation. No abnormal development was observed in these embryos in the stages preceding gastrulation. However, the embryos that were injected with 100 pg Gcri-1 mRNA, but not Gcri-r mRNA, had severe gastrulation problems. When the embryos reached the neurula stage, the normal gravitational orientation of these embryos was reversed. The embryos that had been injected with Gcri-1 mRNA were oriented with the neural plate facing upwards (Fig. 4A), similar to a normal neurula embryo. The neurula embryos that had been injected with Gcri-1 mRNA had their ventral side facing upwards (Fig. 4A). These embryos had a normally developing neural plate (Fig. 4B, indicated with N) and normal neural tube formation (Fig. 4C, indicated with N).

The phenotype created by the injection of Gcri-1 mRNA was due to the abnormal development of the blastocoel. Normally the blastocoel becomes progressively smaller during gastrulation (Fig. 4D). The embryos that were injected with Gcri-1 mRNA, but not Gcri-r mRNA, did not eliminate their blastocoel during gastrulation (Fig. 4E). These embryos, however, did not increase in size and as a consequence the endoderm was pushed against the involuted dorsal mesoderm (Fig. 4E). The combination of the non-reduced blastocoel and the absence of a well-developed archenteron unbalanced the embryo, which subsequently turned upside down (Fig. 4A). In spite of these problems, the embryos that were injected with Gcri-1 mRNA, the migration of the dorsal mesoderm was not disturbed, leading to the normal development of the neural plate (Fig. 4B, C). When the embryos were cultured longer, the blastocoel remained as a blister on the ventral side of the embryo up to stage 20 (Fig. 4C). These data suggest that both Gcri-1 and Gcri-r, but not Gcri-1, have a role in the mechanisms that lead to the obliteration of the blastocoel and concomitant development of the archenteron during gastrulation.

Discussion

In this paper we characterize the developmental expression and the spatial localizations of the transcripts of the Xenopus Gcri-1 and Gcri-r subunits. The maternally expressed genes showed different expression patterns during early development. All three genes were expressed

(data not shown). To test whether pertussis toxin was able to ADP-ribosylate Gcri subunits in vivo, the following experiment was performed. The extent of the in vivo ADP-ribosylation of the Gcri-1 and Gcri-r proteins would be reflected by a decrease in the in vitro ADP-ribosylation of the remaining pool of Gcri-1 and Gcri-r proteins. A dose-dependent

Fig. 3. Effects of injected Gcri-1 and Gcri-r proteins on the neural competence of early gastrula dorsal and ventral ectoderm. (A) Fertilized eggs were injected with 50 pg Gcri-1 and Gcri-r mRNA and cultured to stage-10 early gastrula. Recombinates were made between dorsal mesoderm and stage-10 dorsal or ventral ectoderm (Otte and Moon, 1992). Recombinates were cultured to stage 22 before total RNA was isolated. The expression of neural specific genes in 10 μg RNA was monitored by RNAase protection, using the probes for the neural specific genes XIF3 and N-CAM. The level of S5 transcripts was monitored by RNA blot analysis of these samples. (B) Control embryos (lanes 1 and 3) and embryos that were injected with 50 pg Gcri-1 mRNA (lanes 2 and 4) were cultured to stage 10 before homogenization. ADP-ribosylation was carried out in the presence of pertussis toxin and [32P]NAD+. After labeling, the Gcri-1 and Gcri-r proteins were immunoprecipitated using antibodies against Gcri-1 protein (lanes 1 and 2) or Gcri-r protein (lanes 3 and 4) and separated on an SDS-10% polyacrylamide gel.
almost exclusively in the ectoderm of gastrula embryos and predominantly in the neuroectoderm of neurula embryos. In previous studies, we found that upon neural induction both the protein kinase C (PKC) and the cAMP signal transduction pathways are activated (Otto et al., 1988, 1989, 1990). Given this extensive occurrence of signal transduction processes during neural induction, we investigated a potential role of the Xenopus G-proteins in mediating neural induction. The clearest indication that G-proteins are involved in mediating the initial phases of neural induction comes from our overexpression of the G\(\alpha_{\text{Go}}\) subunit. Overexpression of the G\(\alpha_{\text{Go}}\) subunit increased the neural competence of gastrula ectoderm approximately four-fold. Since G\(\alpha_{\text{Go}}\) activates adenylate cyclase, it is possible that this is an important function of the G\(\alpha_{\text{Go}}\) protein during neural induction. The activation of the G\(\alpha_{\text{Go}}\) protein would lead to the previously observed increases in both the adenylate cyclase activity and cAMP increase during neural induction. The increase in neural competence after overexpressing G\(\alpha_{\text{Go}}\), as well as its localization, is therefore consistent with these earlier observations.

The possible roles of the G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1 genes in neural induction were investigated by overexpression and by the specific inhibition of these proteins by pertussis toxin. Neither the quantitatively limited effects of overexpression of the G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1 subunits nor the inability of pertussis toxin exposure to affect neural competence favor a major role in mediating the initial phases of neural induction. However, overexpression of either the G\(\alpha_{\text{Go}}\) or G\(\alpha_{\text{Go}}\)-1 subunits had a strong inhibiting effect on the normal elimination of the blastocoel during gastrulation. These effects support a role for the G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1 proteins in the normal disappearance of the blastocoel during gastrulation. The effects were highly specific since only overexpression of the G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1, but not the G\(\alpha_{\text{Go}}\)-3 subunits lead to this defect, and we know of no other mRNA that leads to this phenotype upon injection into the fertilized egg. The dose of injected mRNA G\(\alpha_{\text{Go}}\)-1 that led to a four-fold increase in neural competence, had no effect on the elimination of the blastocoel. Conversely, the same dose of injected G\(\alpha_{\text{Go}}\) or G\(\alpha_{\text{Go}}\)-1 mRNA, which had profound effects on the elimination of the blastocoel, had only limited effects on neural competence.

Little, if anything, is known about the mechanisms that underly the obliteration of the blastocoel during gastrulation. It has been hypothesized that the fluid is normally pumped from the blastocoel to the archenteron by an energy-dependent process (Tuft, 1961a, b), but this has been neither proven nor tested. The present data indicate that the normal obliteration of the blastocoel is not simply a passive process, but may be an active process in which G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1 proteins are involved. It is of interest that Cl\(-\) channels, which are involved in regulating Na\(^+\) excretion by renal collecting duct cells, are directly regulated by G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1, but not the G\(\alpha_{\text{Go}}\)-3 proteins (Light et al., 1989a, b). Our data are therefore very suggestive for a role of the G\(\alpha_{\text{Go}}\) and G\(\alpha_{\text{Go}}\)-1 proteins in the regulation of pumps that regulate the volume of the blastocoel.

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