Most Apert syndrome patients harbor a single amino acid mutation (S252W) in fibroblast growth factor (FGF) receptor 2 (FGFR2), which leads to abnormal FGF/FGFR2 signaling. Here we show that specific combinations of FGFRs and glycosaminoglycans activate both alternative splice forms of the mutant but not of the wild-type FGFR receptors. More importantly, 2- and N-sulfated heparan sulfate, prepared by a combined chemical and enzymatic synthesis, antagonized the over-activated FGFR2b (S252W) to basal levels at nanomolar concentrations. These studies demonstrated that specific glycosaminoglycans could be useful in treating ligand-dependent FGFR signaling-related diseases, such as Apert syndrome and cancer.

Apert syndrome is one of the most severe types of human craniosynostosis syndromes. The vast majority of Apert syndrome patients have one of two missense mutations in adjacent amino acids, S252W or P253R, of fibroblast growth factor receptor 2 (FGFR2) (1, 2), occasionally patients have an Alu-element insertion in the FGFR2 gene (3). The S252W mutation, found in two-thirds of Apert patients, has been associated with the most severe craniofacial anomalies and dermatological disorders (4, 5). The P253R mutation is associated with more severe fusion of the bones in fingers and toes (4, 5). Both crystallographic and biochemical studies of FGFR2 (S252W) and (P253R) mutants with various FGFRs indicate that the mutations, in the highly conserved linker region between the immunoglobulin-like domains II and III, resulted in increased affinity and altered specificity of FGF ligand binding (6–9). A mouse model supports the idea that the Apert S252W mutation in FGFR2 renders the mesenchymally expressed mutant FGFR2c abnormally susceptible to activation by mesenchymally expressed FGF7-like ligands and the epithelially expressed mutant FGFR2b abnormally susceptible to activation by epithelially expressed FGFs. Thus, these mutations circumvent the normal reciprocal epithelial-mesenchymal signaling restrictions (10).

Glycosaminoglycans (GAGs) are required for FGF/FGFR signaling at both the cellular and whole animal levels (11–15). Animals defective in GAG biosynthesis have a complete loss of FGF/FGFR signaling (16–18). Two major types of GAGs are heparan sulfate (HS) and chondroitin sulfate (CS). Both HS and CS are highly sulfated linear GAG chains abundantly expressed on the cell surface and in the extracellular matrix in the form of HS, CS, or HS/CS hybrid proteoglycans. FGF/FGFR-GAG interactions depend to a large extent on specific GAG sequences, which can activate, inhibit, or have no effect on FGF/FGFR signaling (19–23).

Apert FGFR2 (S252W) signaling cannot be explained solely by the additional interaction between the mutant receptor and FGFR2 observed in the crystal structure (7). For example, FGF7 promotes mutant FGFR2 (S252W) signaling, but the binding affinity between FGF7 and mutant FGFR2c is negligible (8, 24), possibly because specific GAGs mediate this interaction. The severity of the phenotype varies in Apert patients, although they carry the same FGFR2 (S252W) mutation. Furthermore, only certain tissues and organs are affected during development and in the adult. This may be due to ligand availability and to the modification of activity by specific GAG sequences.

GAG sequences are not directly encoded by genes but are assembled in the Golgi by enzymes encoded by over 40 genes. Because of the vast expression repertoire of the GAG assembly enzymes, GAGs may have a sulfation pattern, chain length, and fine structure unique to each cell (25). Cell surface GAGs turn over within one-eighth to one-third of a cell cycle (26). This means their quantity and fine structures are able to rapidly change in response to a variety of environmental factors.

Heparin and heparin oligosaccharides have been used in most studies of the effect of GAGs on FGF/FGFR signaling. Heparin, the most heavily sulfated HS, is made by mast cells and is usually stored in mast cell granules. The only obvious defect in heparin-deficient mice involves connective tissue-type mast cells (27, 28). In contrast, mice deficient in HS and CS show defects in FGF signaling during gastrulation (15, 17, 18). Therefore heparin is not biologically relevant, but HS and CS are (22, 23, 29). Kidney HS has unique 3-O-sulfated sequences that are different from that of 3-O-sulfated heparin (30).

Altered cartilage and bone development are two of the primary phenotypes associated with the pathogenesis of Apert syndrome (10). Cartilage is enriched in extracellular matrix proteoglycans and contains both HS and CS. CS-A is the most abundant GAG in the growth plate and joints. CS-B, also called dermatan sulfate, is enriched in skin and wound fluid (23). CS-E, a highly sulfated CS, interacts with chemokines and growth factors (31). CS-E inhibits the herpes simplex virus infection better than heparin in a cell-based model system (32).

Because of the essential role of GAGs in regulating FGF activity, we hypothesized that not only HS, but also the abundant CS-A, could differentially modulate the activity of wild-type and mutant FGFR2.
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GAGs—Heparin (H4784), heparan sulfate from bovine kidney (H9637), CS-A from bovine trachea (C8529), and CS-B from porcine intestinal mucosa (C3788) were purchased from Sigma. The major repeating disaccharide (90%) in CS-A is GlcUA-GalNAc4S. CS-B is also called dermatan sulfate. CS-B is enriched in IdoA-GalNAc4S5 repeated disaccharides. Squid cartilage CS-E was purchased from Calbiochem (catalog number 230690). About 60% of the repeating disaccharides in CS-E are GlcUA-GalNAc4S6S. The charge density is heparin → CS-E → CS-B → CS-A → HS.

EXPERIMENTAL PROCEDURES

GAGs—Heparin and heparan sulfate were used in the cell culture medium. Because BaF3 cells express no endogenous FGF, cell survival in IL-3-free medium only if FGF and heparin are both present. BaF3 cells die if only FGF or GAG is included in IL-3-free cell culture medium. Because BaF3 cells express no endogenous FGF, the background activity in the absence of added GAG is very low regardless of whether the cells express an FGFR. BaF3 cells make low levels of GAGs (~74 ng/10^6 cells, ~20% HS (~14 ng/10^6 cells), and ~80% CS (~60 ng/10^6 cells)).

RESULTS

The FGFR2 (S252W) Mutation Altered Specificity for Both FGFs and GAGs—Apert mutant and wild-type FGFR2b- and FGFR2c-expressing cells were used to assay GAG-mediated FGF signaling (8). Wild-type or mutant FGFR2b-expressing BaF3 cells (Fig. 1) were treated with 2 nM FGF2, -7, or -9, and varying concentrations of GAGs (heparin, HS, CS-A, CS-B, or CS-E from 0 to 2 μg/ml). In the absence of GAGs, FGF2, -7, and -9 were unable to activate either wild-type or mutant FGFR2b (see the zero concentration point in each plot, Fig. 1). Consistent with previous studies, wild-type FGFR2b was only activated by FGF7 (8). Relative to wild-type FGFR2b, FGF2b (S252W) was more susceptible to activation induced by 0.125 μg/ml of heparin and FGF7 (90% of the maximum response for the mutant versus 75% for wild-type, Fig. 1, B and E), consistent with increased affinity of FGF7 and mutant FGFR2b (S252W) (8). CS-B (2 μg/ml) and FGF7 (Fig. 1B) also showed a detectable mitogenic activity on wild-type FGFR2b, which is consistent with previous reports (22, 23). However, FGFR2b (S252W) preferentially responded to distinct GAGs in the presence of the three FGFs (Fig. 1, D–F). Cell proliferation was stimulated by FGF9 plus heparin, FGF2 plus heparin or CS-B, and FGF7 plus heparin, CS-B, or HS.

Although the same FGFR2b-expressing BaF3 cells were used in the experiments shown in Fig. 1 (top, wild-type; bottom, mutant), the background [3H]thymidine incorporation in the absence of added GAGs depended on the FGF ligand. FGF2 generated a higher background compared with that of FGF7 and FGF9.

Similar experiments with BaF3 cell lines expressing FGFR2c showed changes in GAG specificity in the presence of the wild-type receptor (Fig. 2, top) and the S252W mutant receptor (Fig. 2, bottom). Wild-type FGFR2c/FGF2 (Fig. 2A) was most efficiently activated by heparin and CS-B, whereas FGFR2c (S252W)/FGF2 was activated by all five GAGs tested (Fig. 2D). In contrast, mutant (Fig. 2E), but not wild-type (Fig. 2B), FGFR2c/FGF7 was activated by heparin. Wild-type FGFR2c/FGF9 (Fig. 2C) was maximally activated by heparin and showed a modest response to CS-B (2 μg/ml). In contrast FGFR2c (S252W)/FGF9 (Fig. 2F) was activated by heparin, CS-B, and HS from bovine kidney. CS-E (2 μg/ml) also showed a detectable mitogenic effect on the mutant-expressing cell line (Fig. 2F). FGFR2c (S252W) required lower doses of GAGs for activation or was less selective with respect to GAGs than wild-type FGFR2c.

The same mutant FGFR2c-expressing BaF3 cells were used in the experiments shown in Fig. 2, D–F. However, the background [3H]thymidine incorporation in the absence of added GAGs was high (FGF2, 200 nM heparin 800 nM GlcN residue. Assuming the commercial heparin has an average molecular mass of 16,000 Da and the repeating disaccharide has a molecular mass of 60 Da, the average chain length should be 16,000/60 = 270 disaccharides, which is equivalent to 27 GlcN residues. Thus, 1 μg/ml of heparin equals 800 nM/27 = 30 nM heparin. Because our chemically and enzymatically synthesized HSs were originally derived from the same heparin, 27 GlcN chains were used to calculate the HS molar concentration. However, the average chain lengths of HS from bovine kidney, CS-A, -B, and -E are unknown. Their concentrations were either expressed as μg/ml in Figs. 1 and 2 or μM GlcN or GalN in Fig. 4. One μg/ml HS equals 871 nM GlcN, 1 μg/ml CS-A is equivalent to 892 nM GalN, 1 μg/ml CS-B = 716 nM GalN, and 1 μg/ml CS-E = 333 nM GalN.

Combined Chemical and Enzymatic Synthesis of HS—Briefly, 2-O- and N-sulfated HS (25SNS HS) was derived from completely desulfated and chemically N-sulfated heparin by enzymatic 2-O-sulfation. 6-O-, 2-O-, and N-sulfated HS (65SNS HS) was obtained by enzymatic 6-O-sulfation of 25SNS HS (36).

4 K. Gilum, D. R. Studelska, and L. Zhang, unpublished results.

80% CS

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32,000 cpm; FGF9, 22,000 cpm; and FGF7, 10,000 cpm). These results suggest that the proliferation was stimulated by intrinsic BaF3 and/or fetal bovine serum GAGs or resulted from FGF-dependent, but GAG-independent, activity. When BaF3 cells treated with both chondroitinase ABC and heparin lyases were assayed in AIM-V medium (which does not contain fetal bovine serum), the background was reduced by ~50% (data not shown). We suspect that other sugar structures, such as undigested GAG linkage oligosaccharides on proteoglycan core proteins, GM1 ganglioside (37), or other N-linked or O-linked sulfated sugars also contribute to the background mitogenic activity.
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The highest GAG concentration used was 2 μg/ml. However, the physiological concentrations of GAGs are generally higher than those used in Figs. 1 and 2 in most in vitro studies. For example, GAG concentrations in wound fluid ranged from 15 to 65 μg/ml (29). CS concentrations in growth plates varied from 10 to 14 mg/ml. The highest heparin concentration was increased from 2 μg/ml (Figs. 1 and 2) to 78 μg/ml.

Heparin activated every FGF (1, 2, 4, 7, 9, or 10) at low concentrations in FGFR2b (S252W)-expressing BaF3 cells (Fig. 4), but the dose-response curve differed from that of other GAGs (HS, CS-A, -B, and -E). FGF1, the universal FGF that activates all of the FGFRs, was activated by all the GAGs tested (at least at the highest concentrations). In contrast, FGF2 was not activated by HS. In fact, [3H]thymidine incorporation decreased with increased HS concentrations. Furthermore, HS at high concentrations inhibited heparin (0.5 μM)-induced [3H]thymidine incorporation of FGFR2b (S252W)-expressing BaF3 cells (data not shown). FGF4 and FGF10 were activated by heparin, CS-A, and CS-B. CS-B activated FGF10 better than CS-A, but CS-A activated FGF4 better than CS-B. CS-A reached the level of heparin activity for FGF4 at a GalN equivalent concentration of 500 μM (data not shown). In contrast, maximum CS-B activity for FGF4 was ~50% that of heparin at a concentration of 100 μM (data not shown). In the presence of FGF7 or FGF9, FGFR2b (S252W)-expressing BaF3 cells were activated by heparin, HS, CS-A, and CS-B. CS-A and CS-B were at the highest concentration, were as effective as heparin in promoting FGF7-induced proliferation of FGFR2b (S252W)-expressing BaF3 cells. However, with FGF9, CS-A and CS-B were only 60% as effective as heparin at the highest concentrations.

Inhibition of FGFR2b (S252W) by Modified HS—The mitogenic activation and inhibition of 6-O-desulfated heparin and the inhibition of 2-O-sulfate-enriched heparin oligosaccharides toward specific FGF/FGF signaling have been reported (19–21, 38, 39). In the presence of FGF7 or FGFR2b (S252W)-expressing BaF3 cells were activated by heparin, HS, CS-A, and CS-B. CS-A and CS-B are mixtures of GAG produced by different kinds of cells (in bovine kidney or squid cartilage). We therefore decided to search for structurally defined polysaccharide inhibitors. By a combined chemical and enzymatic approach, we synthesized 2SNS HS and 6S2SNS HS (36). Synthetic 2SNS HS inhibited FGFR2b (S252W) activity in a dose-dependent manner (Fig. 5). The first bar in both panels represents activation by intrinsic GAGs or other cofactors in combination with FGF2 (top) or FGF7 (bottom). When 2 nM FGF2 and 15 nM heparin were used in the assay (Fig. 5, Top), 60 nM 2SNS HS reduced the [3H]thymidine incorporation by 18%, whereas 120 nM 2SNS HS showed nearly complete inhibition (98%) of FGF2b (S252W) activity. This is comparable to the [3H]thymidine incorporation data in the absence of FGF2 (Fig. 5, heparin 15 nM), indicating that 2SNS HS not only inhibited the activity of heparin but also of other intrinsic GAGs or other cofactor activities that gave the higher [3H]thymidine incorporation in the absence of heparin (Fig. 5, FGF2 2 nM). 6S2SNS HS had no inhibitory activity, even though it was made by 6-O-sulfation of 2SNS HS. In fact, 6S2SNS HS stimulated [3H]thymidine incorporation at both 60 and 120 nM concentrations (Fig. 5, top).

When 2 nM FGF7 was used (Fig. 5, bottom), inhibition by 2SNS HS was also observed. 2SNS HS (60 nM) reduced the [3H]thymidine incorporation by 16%. Doubling the 2SNS HS to 120 nM inhibited the [3H]thymidine incorporation by 84%. Again 6-O-sulfation of 2SNS HS...
eliminated its inhibitory activity but did not stimulate [3H] thymidine incorporation (Fig. 5, bottom).

**DISCUSSION**

The Requirement of "Specific GAGs" in Activating FGF Signaling—Our results were novel in the following aspects. 1) We showed that CS-A and CS-E activated FGF/FGFR signaling at biologically relevant concentrations (Figs. 2 and 4). 2) CS-B was not restricted to activate wild-type FGFR2b with FGF2 or FGF7 as reported in the past (22, 23, 29). It also activated wild-type FGFR2c (Fig. 2) and FGFR2b (S252W) with all FGFs (1, 2, 4, 7, 9, and 10) tested (Fig. 4). 3) HS from bovine kidney activated mutant FGFR2b with FGF1, but inhibited mutant FGFR2b with FGF2 (Fig. 4 and "Results"). 4) Most importantly, chemically and enzymatically synthesized 2SNS HS antagonized the over-activated FGFR2b (S252W) to basal level at nanomolar concentrations.

Sugar Specificity Involved in FGF/FGFR Signaling—Of all the GAGs we tested, heparin possesses the structural features that initiate maximum BaF3 cell proliferation (Figs. 1–4). But biologically irrelevant sulfated polysaccharides, such as dextran sulfate and fucoidan, work better than heparin in initiating specific FGFR signaling (40). Usually octa-oligosaccharides and larger heparin oligosaccharides are required for FGF/FGFR signaling, but disaccharides or trisaccharides have been reported to have activity in FGF/FGFR signaling (41, 42). Cell membrane ganglioside GM1, another type of sugar, was found to facilitate FGF2/FGFR1c-mediated cell proliferation (37). Without detailed knowledge of assay systems, especially the specific FGF or FGFR involved in the referenced studies, the contribution of sugar specificity is difficult to define. At physiological concentrations of GAGs, each FGF showed a unique receptor activation capability (Fig. 4). Bovine kidney HS promoted mutant FGFR2b/FGF1 signaling (Fig. 4, FGF1) but did not activate FGFR2b (S252W)/FGF2 signaling (Fig. 4, FGF2). CS-A and CS-B could attain the activation level of heparin, but HS could only attain 60% of the activation level of heparin even at the highest concentrations tested (Fig. 4, FGF7). These observations revealed that specific GAG, FGF, and FGFR combinations determine the level of ligand-dependent FGF signaling and suggest that cell and tissue specific GAG structures and their local concentrations are important regulators of FGF/FGFR activity.

Our Data and the Current FGF-FGFR-GAG Models—The discovery that HS forms a ternary complex with FGF and FGFR (12, 13, 43) led to numerous models of the molecular mechanism by which a GAG enables FGF and FGFR to assemble into an active dimeric complex (44). Two models have dominated the field because two FGF/FGFR/GAG co-crystal structures were solved. One model proposed a symmetric structure consisting of 2 FGF2:2 FGFR1c:2 heparin non-reducing ends. The co-crystal was obtained by diffusing heparin decasaccharides into the binary FGF2/FGFR1c crystal (45). The other model proposed an asymmetric structure composed of 2 FGF1:2 FGFR2c:1 heparin (46). This co-crystal was directly formed from a solution containing FGF1, FGFR2c, and heparin decasaccharide. The difference between the two co-crystals was that in the first crystal, FGF2 and FGFR1c interact and form a binary crystal prior to GAG addition, whereas in the second co-crystal, GAG facilitates FGF1 and FGFR2c interaction because FGF1 and FGFR2c do not interact with each other (47). In the co-crystal structures, proline (253), in the highly conserved linker region of FGFR,
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adopted a trans-conformation in the symmetric two ends co-crystal and a cis-conformation in the asymmetric co-crystal.

Much experimental data supports the symmetric "two ends" model in which "the heparin-binding sites of FGF and FGFR merge to form a basic canyon that recruits two HS for binding. Within this canyon, the HS molecules primarily act to orchestrate and fortify multivalent and cooperative protein-protein contacts within the dimer that are the foundations of dimerization" (48). The high affinity interaction between FGF and FGFR forms the basis of the two ends model. In this model, the role of GAG is secondary, so even a disaccharide (octasulfated sucrose) or HS trisaccharide can activate FGF/FGFR-mediated mitogenic activities (41, 42). The co-crystal structure of Apert syndrome FGFR2 (S252W)/FGF2 was consistent with the FGFR1c/FGF2 co-crystal structure on which the two ends model was based (7).

Because of the increased FGF/FGFR2 (S252W) affinity, the two ends model would predict a lower specificity for GAG structures. The observation that the mutant FGFR can be activated not only by heparin, but also by CS-A, -B, -E, and HS, is consistent with the two ends model. How- ever FGF7 was activated more efficiently by heparin, CS-A, -B, and HS than FGF9 (Fig. 1, E and F, and Fig. 4, FGF7 and FGF9). Thirdly, FGF7 had lower affinity ($K_d = 1.04 \times 10^{-5}$ M) for the wild-type FGF2b than did FGF4 ($K_d = 5.34 \times 10^{-7}$ M) (24). However, FGF7 activated wild-type FGF2b (Fig. 1B), but FGF4 did not activate FGF2b (49). These results suggest that high affinity FGF/FGFR interactions are not a prerequisite for forming an active FGF-FGFR-GAG signaling complex.

Alternate Explanation for Activation of FGFR2 (S252W) Signaling in Apert Syndrome—The amino acid residues in the linker region (RSPHRPR) are highly conserved in almost all FGFRs. Apert mutations yield new linker peptide sequences of RWPHPR and RSRHRP. The Apert peptides could enhance interactions with GAGs because of a stretch of basic and aromatic amino acid residues. The interaction might induce conformation changes in FGFR and affect the cis- or trans-conformation of the FGFR observed in two distinct FGF-FGFR-GAG co-crystals (45, 46). However, FGF, but not GAG, interacts with the FGFR linker region in both FGF-FGFR-GAG co-crystal structures. This suggests a model in which FGF-FGFR-GAG ternary complex formation parallels the heparin/thrombin/antithrombin anti-coagulant system (50). Heparin interacts with both thrombin and anti-thrombin. This interaction causes conformational changes and accelerates thrombin/antithrombin complex formation by 1000-fold. However, once the binary thrombin and antithrombin complex is formed, heparin dissociates from the complex (50). In our model, the Apert mutation may accelerate the GAG and FGFR2 interaction because such an interaction has been proposed to be the rate-limiting step in forming a functional FGF-FGFR-GAG signaling complex (51). This interaction may cause a conformation change in FGFR2 and allow a functional FGFR2-FGF-GAG ternary complex to form. However, experiments are needed to test our hypothesis.

Molecular Basis for the Inhibitory Effect of the 2-O- and N-Sulfated HS—Our studies using a biochemical assay showed that certain cellular HS interact with FGFR1 in a cation-dependent manner. Such an interaction may inhibit FGF1/FGF interactions (52). Based on this study, 2SNS HS inhibition might be through interaction with mutant FGFR2b.

We and others (44, 53) previously showed that although HS does not interact with FGF or FGFR alone, it forms a FGF-FGFR-HS ternary complex and has mitogenic activity. Although 2SNS HS has a lower affinity for both FGF and FGFR, we cannot eliminate the possibility that a ternary complex incapable of FGFR dimerization was formed in the presence of 2SNS HS.

Further studies are needed to test each possible inhibitory mechanism imposed by 2SNS HS. Understanding such inhibitory mechanisms is critical for rational drug design.

2-O- and N-Sulfated HS Resembles Physiologically Relevant HS Structures—Recent studies show that following its synthesis and expression, HS can be structurally and functionally modified within the extracellular compartment (54, 55). For example, the HS 6-O-endosulfatasase-generated HS should have structures similar to our 2-O- and N-sulfated HS. Indeed, HS 6-O-endosulfatasase inhibits FGF signaling in mesoderm induction and angiogenesis in Drosophila (58) and inhibits myeloma tumor growth in SCID mice (59) as a result of removing 6-O-sulfates from HS.

Developing a GAG Drug That Regulates FGF/FGFR Signaling—The FGF complex represents an excellent target for chemotherapy of Apert syndrome, cancers, and other related diseases because of hyper- or hypo-FGF signaling. FGF-FGFR-GAG interactions are highly specific.
and regulated, so an agent could be administered with a lower risk of side effects. Additionally, the site of action of a GAG inhibitor is outside the cell, which removes the difficulty of crossing a biological membrane and reduces the likelihood of drug resistance. However, even with a good model of FGF signal transduction, the rational design of GAG or other inhibitors is a process fraught with difficulties.

**Conclusion**—The regulation of FGF/FGFR signaling by GAGs is an important but poorly studied area. The inhibition or activation of FGFR signaling promoters/inhibitors or as an antiangiogenesis drugs (19–21, 22) has been tested in different FGF/FGFR signaling assays as well as in assays (19, 23) of the same FGF/FGFR pair, e.g. FGF2/FGFR1C, in biological assays (19, 24). We showed that 2-O- and N-sulfated HS derived from heparin by a combined chemical and enzymatic synthesis approach inhibits FGF2b (S252W) signaling. The degree of sulfation imposed by sulfotransferase is a process fraught with difficulties. Good model of FGF signal transduction, the rational design of GAG or other inhibitors is a process fraught with difficulties. N-Desulfated, 2-O-desulfated, 6-O-desulfated heparin, and other chemically modified heparins have been tested in different FGF/FGFR signaling assays as signaling promoters/inhibitors or as an antiangiogenesis drugs (19–21, 24). We showed that 2-O- and N-sulfated HS derived from heparin by a combined chemical and enzymatic synthesis approach inhibits FGF2b (S252W) signaling. The degree of sulfation imposed by sulfotransferase is moderate, which gives specificity and avoids over-sulfation-induced nonspecific interactions with other proteins.

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