MOLECULAR AND CELLULAR BIOLOGY, Aug. 1998, p. 4689–4697 Vol. 18, No. 8
0270-7306/98/$04.00+0

CDC42 and FGD1 Cause Distinct Signaling and Transforming Activities

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Received 9 January 1998/Returned for modification 6 March 1998/Accepted 29 May 1998

Activated forms of different Rho family members (CDC42, Rac1, RhoA, RhoB, and RhoG) have been shown to transform NIH 3T3 cells as well as contribute to Ras transformation. Rho family guanine nucleotide exchange factors (GEFs) (also known as Dbl family proteins) that activate CDC42, Rac1, and RhoA also demonstrate oncogenic potential. The faciogenital dysplasia gene product, FGD1, is a Dbl family member that has recently been shown to function as a CDC42-specific GEF. Mutations within the *FGD1* locus cosegregate with faciogenital dysplasia, a multisystemic disorder resulting in extensive growth impairments throughout the skeletal and urogenital systems. Here we demonstrate that FGD1 expression is sufficient to cause tumorigenic transformation of NIH 3T3 fibroblasts. Although both FGD1 and constitutively activated CDC42 cooperated with Raf and showed synergistic focus-forming activity, both quantitative and qualitative differences in their functions were seen. FGD1 and CDC42 also activated common nuclear signaling pathways. However, whereas both showed comparable activation of c-Jun, CDC42 showed stronger activation of serum response factor and FGD1 was consistently a better activator of Elk-1. Although coexpression of FGD1 with specific inhibitors of CDC42 function demonstrated the dependence of FGD1 signaling activity on CDC42 function, FGD1 signaling activities were not always consistent with the direct or exclusive stimulation of CDC42 function. In summary, FGD1 and CDC42 signaling and transformation are distinct, thus suggesting that FGD1 may be mediating some of its biological activities through non-CDC42 targets.

The Rho subfamily of Ras-related GTPases (14 mammalian members) controls multiple aspects of cell behavior, including cytoskeletal rearrangement, nuclear signaling, and cell growth (reviewed in reference 67). For example, CDC42 mediates the induction of actin microspikes and filopodia by bradykinin (26, 33), whereas Rac1 is required for growth factor-induced membrane ruffling and lamellipodia formation (47). In contrast, RhoA regulates the formation of actin stress fibers (46). In Swiss 3T3 cells, the assembly of these structures involves a cascade in which CDC42 activates Rac1, which in turn activates RhoA (33). Rho family proteins also have demonstrated roles in the regulation of gene expression as measured by (i) the transcriptional activation of the serum response factor (SRF) (19), (ii) activation of c-Jun NH_2-terminal kinase (JNK) and its downstream target c-Jun (10, 32, 35), (iii) activation of the ternary complex factor protein Elk-1 (62), (iv) activation of p38/Mpk2 (63), and (v) regulation of expression from the cyclin D1 promoter (55). Finally, there is growing evidence that the deregulated expression of Rho family members has profound effects on the proliferative potential of cells. Activated derivatives of RhoA, RhoB, Rac1, and CDC42 cause oncogenic transformation when expressed in rodent fibroblast cell lines and may contribute to Ras-mediated malignant transformation (23, 39, 41, 42, 53, 67).

Rho family GTPases function as regulated switches that cycle between a biologically active GTP-bound and an inactive GDP-bound form (5). They are activated by guanine nucleotide exchange factors (GEFs) that catalyze the exchange of bound GDP for GTP and inactivated by GTPase-activating proteins that stimulate GTP hydrolysis (4). The Dbl-related proteins are a large family of structurally related molecules that have a common ability to catalyze GEF activity for specific members of the Rho family (7, 59). Like activated derivatives of their putative GTPase targets, catalytically active derivatives of many Dbl-related proteins are highly oncogenic, promoting tumor growth in nude mice.

The region of sequence similarity that defines members of the Dbl family consists of a Dbl homology (DH) domain arranged in tandem with a pleckstrin homology (PH) domain. An intact DH domain is essential for the GEF activity of the Dbl protein (the mammalian prototype of the Dbl family) as well as for the transforming activity of many Dbl family proteins (20, 31, 48, 57, 58, 60). The PH domain also mediates the transforming activity of Dbl-related proteins, in part, by targeting them to specific cellular locations (58, 65).

FGD1 was determined by positional cloning to be the gene responsible for faciogenital dysplasia (FGDY) (also known as Aarskog-Scott syndrome), an X-linked skeletal dysplasia first described in 1970 (1). Mutations at the FGDY locus alter the size and shape of a number of small bones and cartilage elements but leave other skeletal structures unaffected (15, 16). The cardinal features of this disease include widely spaced eyes (hypertelorism), ptosis, down-slanting palpebral fissures, dysplastic ears, maxillary hypoplasia, and disproportionate acromelic short stature; radiographic abnormalities include maxillary and mandibular hypoplasia, hypoplastic phalanges, retarded bone maturation, and a variety of vertebral anomalies including cervical spina bifida occulta and odontoid hypoplasia (15, 16). These observations suggest that, like the genes responsible for the mouse mutations short ear (25) and brachypodism (50), *FGD1* acts on a limited number of mesenchymal condensations during skeletogenesis (16).

The FGDY gene product (FGD1) encodes tandem DH and

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PH domains (38) and has been shown recently to encode GEF activity specific for CDC42 (64). Microinjection of FGD1 into Swiss 3T3 cells induces the formation of filopodial extensions consistent with the in vivo activation of CDC42 (36). The demonstrated relationship between FGD1 and CDC42 function suggests that they may have a common ability to regulate signaling pathways that influence cell growth, cell cycle progression, and transcription.

Although CDC42 involvement in the regulation of cell morphology and gene expression has been well documented (10, 19, 26, 33), its contribution, if any, to proliferative signaling pathways remains unclear. The stable expression of the constitutively activated, GTPase-defective CDC42(12V) mutant has been shown to be growth inhibitory in NIH 3T3 cells (28) yet growth promoting in Rat-1 cells (40). In contrast, a recent report indicates that an NIH 3T3 cell line that has been stably transfected with a second constitutively activated CDC42 mutant [CDC42(F28L)] exhibits many of the hallmarks of transformation (28). This suggests that constitutively activated mutants of CDC42 do not have equivalent activities in biological assays and that they do not necessarily mimic activation of endogenous CDC42 by a GEF. The latter point is illustrated by our recent observation that Dbl family members whose in vitro exchange activities include CDC42 exhibit potent focus-forming activity in NIH 3T3 cells whereas activated derivatives of their putative GTPase targets do not ((56); unpublished observations). Thus, it is unclear whether FGD1 would regulate signaling pathways leading to cell proliferation and transformation or pathways that trigger growth inhibition.

In the present study we have compared the abilities of activated derivatives of CDC42 and FGD1 to transform NIH 3T3 mouse fibroblasts and their abilities to stimulate the activation of nuclear signaling pathways. NIH 3T3 cells were used in these studies, because they are an embryonic fibroblast cell line and our in situ analyses have revealed that the highest level of FGD1 expression occurs during development in mesenchymal condensations (unpublished observations). FGD1 expression is first detected prior to the differentiation of chondrocytes and osteoblasts, when mesenchymal condensations are comprised almost exclusively of embryonic fibroblast cells (17). Whereas both FGD1 and CDC42 can cause growth transformation of NIH 3T3 cells, they do so in qualitatively and quantitatively distinct manners. Similarly, both FGD1 and CDC42 exhibit distinct signaling profiles when assayed for their ability to stimulate the activation of nuclear signalling pathways mediated by SRF, Elk-1, and c-Jun. Although the stimulation of these transcriptional activities by FGD1 occurs in a CDC42-dependent manner, they cannot always be attributed to direct or exclusive stimulation of CDC42 function. In summary, our comparison of CDC42 and FGD1 transforming and signaling activities suggests that FGD1 may regulate some of these activities through non-CDC42 targets.

MATERIALS AND METHODS

Molecular constructs. pRK5-myc-*FGD1* (36) encodes the tandem DH and PH domains of the FGD1 protein (residues 375 to 710) fused at the NH_2 terminus to a Myc epitope tag. pRK5-myc-*FGD1*Δ (36) encodes a naturally occurring splice variant of *FGD1* that contains a deletion within the DH domain; it encodes a derivative with the same NH_2 and COOH termini as pRK5-myc-*FGD1* but is missing residues 398 to 433 (37). pAX142-myc-*FGD1* and pAX142-myc-*FGD1*Δ were made by isolating the *Sca*I/*Eco*RI inserts from pRK5-myc-*FGD1* and pRK5-myc-*FGD1*Δ, respectively, filling in the ends of the fragments with T4 DNA polymerase, and ligating into pAX142 (58) digested with *Sma*I. pAX142-*lsc-D7HA* (56) and pAX142-*Dbl-HA1* (56) encode transforming derivatives of the Lsc and Dbl proteins, respectively, fused in frame at the NH_2 terminus to an epitope from the hemagglutinin (HA) protein of influenza virus. The cDNA sequences encoding wild-type Rac1 and RhoA, dominant-inhibitory RhoA [RhoA(19N)], and dominant-inhibitory CDC42 [CDC42(17N)] were removed from the pZIP-NeoSV(x)1 vector (24) and inserted into the *Sma*I site of pAX142. pAX142-*cdc42(12V)* encodes a constitutively activated derivative of the CDC42 protein (kindly provided by R. Cerione) inserted into the *Sma*I site of pAX142. pcDNA3-HA-*cdc42* encodes wild-type CDC42 protein fused at the NH_2 terminus to an HA epitope (kindly provided by S. Bagrodia and R. Cerione). pZIP-*raf*(*340D*) (24) encodes a weakly transforming, activated derivative of the Raf-1 kinase. pyDF30-*WASP-GBD* encodes the GTPase binding domain (GBD) of WASP (the product of the Wiskott-Aldrich syndrome locus) and was kindly provided by M. Symons (40). WASP was shown recently to be a CDC42-specific effector (3, 52). The cDNA sequences encoding β-galactosidase were removed from pcDNA3.1/His/*lacZ* (Invitrogen) and inserted into the *Sma*I site of pAX142.

Cell culture, transfection, and transformation assays. NIH 3T3 cells were maintained in Dulbecco's modified Eagle medium (high glucose) supplemented with 10% calf bovine serum. Primary focus formation assays were performed in NIH 3T3 cells exactly as described previously (9). Briefly, NIH 3T3 cells were transfected by calcium phosphate coprecipitation in conjunction with a glycerol shock. Focus formation was scored at 14 days. Cognate empty vectors for each construct were employed as controls. NIH 3T3 cell lines that stably express pRK5, pRK5-myc-*FGD1*, and pRK5-myc-*FGD1*Δ were generated by calcium phosphate coprecipitation followed by selection for 14 days in growth medium supplemented with G418 (200 μg/ml). Multiple drug-resistant colonies (>100) were pooled together to establish cell lines for the transformation assays. For secondary focus assays 10^3 stably selected cells were mixed with 10^6 untransfected NIH 3T3 cells and then plated on 60-mm-diameter dishes. Foci were scored at 7 days. Anchorage-independent growth and tumorigenicity in nude mice were measured as described previously (9, 34). For soft agar assays and primary and secondary focus formation assays, experiments were performed in triplicate.

Transient-expression reporter gene assays. For transient-expression reporter assays, NIH 3T3 cells were transfected by calcium phosphate coprecipitation, allowed to recover for 30 h, and starved in Dulbecco's modified Eagle medium with 0.5% newborn calf serum for 14 h before lysate preparation (9, 18, 54). Analysis of luciferase expression in transiently transfected NIH 3T3 cells was performed as previously described by using enhanced chemiluminescence reagents and a Monolight 2010 luminometer (Analytical Luminescence, San Diego, Calif.) (18). The reporter constructs utilized have been described previously: Gal4-Elk-1 (30) and 5×Gal4-luc (51), Gal-Jun(1-223) (51), $(SREm\text{-})_2$Luc (55), and CD1-Luc (2). β-Galactosidase activity in transiently transfected NIH 3T3 cells was determined exactly as described previously (29). All assays were performed in triplicate.

Western blotting. Expression of Myc-epitope-tagged FGD1 proteins in transiently transfected 293 cells and in stably selected NIH 3T3 cell lines was determined by Western blot analysis as described previously (58) by using the 9E10 antibody (Santa Cruz Biotech). Membranes were incubated with horseradish peroxidase-labeled anti-mouse secondary antibodies, and protein was visualized with enhanced chemiluminescence reagents (Amersham).

RESULTS

FGD1 and Raf cooperate to transform NIH 3T3 mouse fibroblasts. Although FGD1 expression alters the actin cytoskeleton in a manner consistent with the in vivo activation of CDC42 (36), its role, if any, in the regulation of cell proliferation has not yet been determined. To investigate the role of the FGD1 protein in the control of cell growth, we compared the effects of expressing catalytically active derivatives of FGD1 and CDC42 on the proliferative properties of NIH 3T3 mouse fibroblasts. During murine development FGD1 expression is first detected in cellular condensations that are comprised exclusively of fibroblasts (unpublished observations). pAX142-myc-*FGD1* encodes a fragment of the FGD1 protein that exhibits in vitro GEF activity specific for the CDC42 protein (64) and induces the formation of filopodia when injected into Swiss 3T3 cells (36). pAX142-*cdc42*(*12V*) encodes an activated derivative of CDC42 that has been shown previously to be transforming in Rat-1 but not NIH 3T3 cells. To assess the relationship between GEF and transforming activity, we also examined the transforming properties of pAX142-myc-*FGD1*Δ, which encodes a biologically inactive peptide derived from a naturally occurring splice variant of the FGD1 protein (37). FGD1Δ is identical to FGD1 except that it harbors a small deletion (residues 398 to 433) within the DH domain.

Initially, we compared the abilities of FGD1, FGD1Δ, and CDC42(12V) to induce the formation of foci in a primary focus

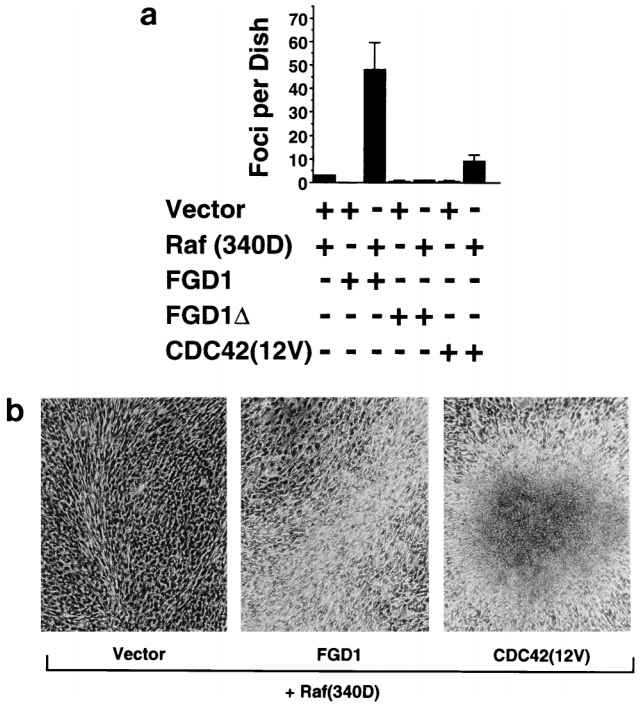

FIG. 1. CDC42(12V) and FGD1 cooperate with Raf-1 in NIH 3T3 focus formation assays. (a) NIH 3T3 cells were cotransfected with pZIP-NeoSV(x)1 or pAX142 expression plasmids encoding the indicated proteins. Five hundred nanograms of each construct was transfected per 60-mm-diameter dish. Foci of transformed cells were counted at 14 days. Data shown are from one experiment performed in duplicate and are representative of three independent experiments. Error bars, standard errors. (b) FGD1 and CDC42(12V) cooperate with Raf(340D) to cause transformed foci with distinct morphologic appearances. Foci were photographed under phase microscopy at 14 days.

formation assay. At DNA concentrations up to 5 μg/60-mm-diameter plate, pAX142-*cdc42*(*12V*), pAX142-myc-*FGD1*, and pAX142-myc-*FGD1*Δ failed to induce focus formation when transfected into NIH 3T3 cells (Fig. 1a). In this respect, FGD1 differs from other Dbl family members that encode GEF activity for CDC42, such as the Dbl and Dbs oncoproteins (56). Activated derivatives of Dbl and Dbs exhibit potent transforming activity in primary focus formation assays (56).

We and others have shown that activated derivatives of the Dbl and Rho families can synergistically interact with activated Raf-1 to induce potent focus formation in NIH 3T3 cells (22, 24, 41, 42). In addition, it has been shown that dominant-inhibitory versions of CDC42, Rac1, RhoA, RhoB, and RhoG can block the transforming activity of Ras (23, 27, 39–42, 49). To further investigate the oncogenic potential of FGD1, we compared the abilities of FGD1 and CDC42 to cooperate with Raf-1 in an NIH 3T3 cell focus formation assay. For this analysis we utilized a weakly activated derivative of Raf-1 [Raf(340D)] that has been shown previously to have very weak activity in a focus formation assay (24). Whereas the expression of either FGD1 or Raf(340D) alone produced relatively few foci, coexpression of FGD1 and Raf(340D) caused a >15-fold enhancement of focus-forming activity (Fig. 1a). A much weaker cooperativity (threefold) was observed when we coexpressed Raf(340D) with CDC42(12V), and no cooperativity was observed between Raf(340D) and FGD1Δ or the empty pRK5 vector control.

Interestingly, the morphology of the foci induced by Raf in cooperation with FGD1 (Raf + FGD1-induced foci) was strikingly different from the morphology of the Raf + CDC42-induced foci (Fig. 1b). The Raf + FGD1-induced foci had a swirled morphology, contained elongated and highly refractile cells, and were indistinguishable in appearance from foci induced by activated Raf or Ras alone (data not shown). In contrast, Raf + CDC42-induced foci lacked refractile cells and exhibited the more dense, stellate morphology reminiscent of transformed foci induced by Rho and Dbl family proteins. Based on the clear differences we observe between FGD1 and CDC42 (both quantitative and qualitative) in these cooperation assays, we conclude that either the *cdc42*(*12V*) mutation does not precisely mimic activation of CDC42 by FGD1 or the FGD1 protein has an in vivo signaling activity that is distinct from CDC42 activation.

Stable expression of FGD1 in NIH 3T3 cells is sufficient to cause anchorage independence and tumorigenic transformation. To further examine the effects of FGD1 expression on cell growth, we established stable lines of NIH 3T3 cells that constitutively express FGD1. NIH 3T3 cells were stably transfected with pRK5-myc-*FGD1*, pRK5-myc-*FGD1*Δ, or the empty pRK5 vector control, and then multiple G418-resistant colonies were pooled. Numerous G418-resistant colonies were

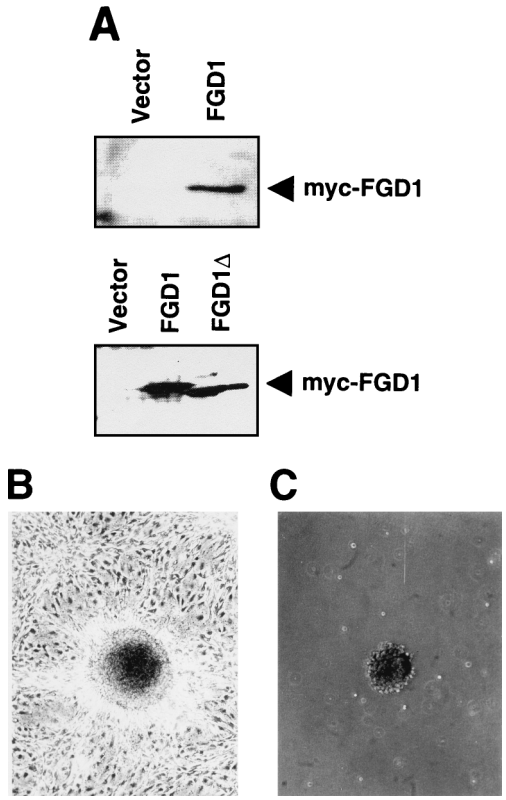

FIG. 2. FGD1 causes growth transformation of NIH 3T3 cells. NIH 3T3 cells were transfected with 3 μg of pRK5 or pRK5-myc-*FGD1* and then selected for 14 days with G418-containing growth medium (200 μg/ml). (a) Expression of FGD1 and FGD1Δ. The upper panel shows FGD1 expression in a stably selected NIH 3T3 cell line. The lower panel shows transient expression of FGD1 and FGD1Δ in 293 cells. Expression was determined by Western blot analysis using an anti-Myc antibody (9E10; Santa Cruz Biotech). (b) NIH 3T3 cells that constitutively express FGD1 cause Rho-like transformed foci in a secondary focus formation assay. Stably selected cells (10^3) were mixed with 10^6 untransformed cells and then plated. Foci were counted and photographed at 7 days. (c) FGD1 promotes anchorage-independent growth in soft agar when constitutively expressed in NIH 3T3 cells. The FGD1-transfected cell line was seeded at 5×10^4 cells per 60-mm-diameter dish in growth medium containing 0.3% agar over a base layer of 0.6%. Colonies were counted and photographed at 21 days.

TABLE 1. FGD1 causes tumorigenic transformation of NIH 3T3 cells

Plasmid	Focus-forming activity (%)[a]	Soft agar growth (%)[b]	Tumorigenicity (freq)[c]
Vector	0.1	0.0	0/6
FGD1	6.8	0.2	5/6

[a] NIH 3T3 cells were transfected with 3 μg of pRK5-myc plasmid or pRK5-myc-*FGD1* and selected for 14 days with G418 (200 μg/ml). Protein expression was determined by Western blotting using an anti-myc antibody (9E10; Santa Cruz). Stably selected cells (10^3) were mixed with 10^6 untransfected cells and then plated. Foci were scored at 7 days. Data are presented as the percentage of transfected cells that gave rise to foci. Data shown are representative of three independent assays done with duplicate plates.

[b] To assess colony formation in soft agar, each transfected cell line was seeded at 5×10^4 cells per 60-mm-diameter dish in growth medium containing 0.3% agar over a base layer of 0.6%. Colonies were counted at 21 days and expressed as a percentage of plated cells. Data shown are representative of three independent assays done in duplicate.

[c] Tumorigenic potential of the transfected cells was determined by subcutaneous inoculation into athymic nude mice (10^6 cells per site). For each cell line, three mice (two sites per mouse) were inoculated. Data are presented as total number of tumors per total number of sites. Mice that were negative for tumor growth were monitored for 3 months before being sacrificed. freq, frequency.

selected from FGD1-transfected cells, and we were able to readily detect expression of FGD1 protein (Fig. 2A). In contrast, relatively few G418-resistant colonies were obtained from the FGD1Δ-transfected cells and we were unable to select cell populations in which we could detect expression of the protein. The failure to detect expression of FGD1Δ was not due to inherent instability of the protein, since good expression was observed in transiently transfected 293 cells (Fig. 2A). A more likely explanation is that FGD1Δ is either toxic or growth inhibitory when constitutively expressed in NIH 3T3 cells.

Although cell populations expressing FGD1 exhibited no obvious morphological transformation compared to cells transfected with vector alone (not shown), the FGD1-expressing cell line exhibited significant secondary focus formation and growth in soft agar (Table 1 and Fig. 2B and C). The focus morphology associated with FGD1 expression (Fig. 2B) is typical of Dbl family members and is reminiscent of the focus morphology that is associated with activated derivatives of RhoA or Rac1 in NIH 3T3 cells (23). We also examined the FGD1-expressing cells for tumorigenicity in nude mice. Cells expressing FGD1 showed rapid tumor formation, while vector control lines did not (Table 1). We conclude that the expression of a catalytically active derivative of FGD1 disrupts the growth properties of NIH 3T3 fibroblasts and is sufficient for tumorigenic transformation.

FGD1 and CDC42 show differing abilities to activate the SRF, Elk-1, and c-Jun transcription factors. The distinct biological activities of FGD1 and CDC42 seen in the Raf cooperativity studies suggested that their signaling activities may also be distinct. To address this question, we compared the abilities of FGD1 and CDC42 to activate the c-Jun and Elk-1 transcription factors and to stimulate transcription from the cyclin D1 promoter. We also compared their abilities to stimulate transcription from a mutant serum response element (from the c-*fos* promoter) that is only responsive to SRF activation. We have shown previously that Dbl family members (including those with GEF activity for CDC42) have a common ability to activate these four transcriptional reporters (56; unpublished observations).

Both FGD1 and CDC42(12V) stimulated transcription from the SRF, c-Jun, and Gal-Elk reporters but showed negligible activation of the cyclin D1 reporter (Fig. 3). Under identical

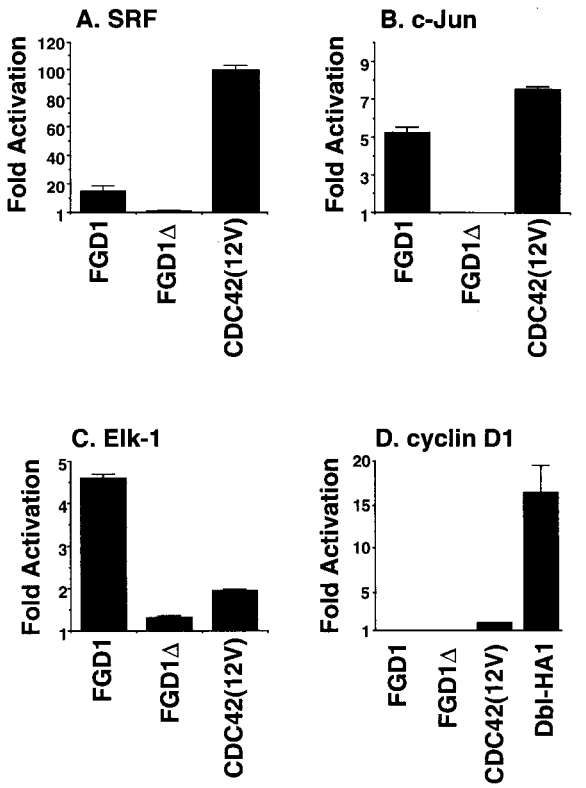

FIG. 3. FGD1 and CDC42 stimulate transcription from common promoter elements. NIH 3T3 cells were transfected with 1.5 μg of pAX142, pAX142-myc-*FGD1*, pAX142-myc-*FGD1*Δ, or pAX142-*cdc42*(*12V*) along with luciferase gene reporter constructs for SRF transcriptional activity (2.5 μg) (A), c-Jun transcriptional activity (500 ng of Gal-Jun and 2.5 μg 5×Gal) (B), Elk-1 transcriptional activity (500 ng of Gal-Elk and 2.5 μg of 5×Gal) (C), or cyclin-D1 transcription (2.5 μg of CD1-luciferase) (D). Following transfection, cells were cultured for 30 h and then serum starved (0.5% calf serum) for 14 h before extract preparation. Luciferase activity was determined and expressed as fold activation relative to the level of activation seen with the vector controls. Data shown are representative of three independent experiments performed on duplicate plates. Error bars, standard errors.

conditions, good activation of cyclin D1 transcription was observed with an activated derivative of Dbl (Dbl-HA1), which is in accordance with our previous observations (56). Although FGD1 and FGD1Δ are expressed at equivalent levels when transiently expressed (Fig. 2A), in no instance did we observe signaling activity associated with FGD1Δ, thus indicating the dependence of these signaling events on FGD1-encoded GEF activity. Although both CDC42 and FGD1 stimulated the same reporters, their relative degrees of activity differed for each assay. Stimulation of SRF by CDC42 was consistently 10-fold higher than that by FGD1, whereas both exhibited roughly equivalent stimulation of c-Jun. In contrast, FGD1 consistently stimulated higher levels of Elk-1 activity (threefold) than that by CDC42. This suggests that the stimulation of these reporters by FGD1 and CDC42 may not be attributable to a common mechanism of activation and again suggests that FGD1 may encode biological activities that are not mimicked by the CDC42(12V) mutant protein.

FGD1 cooperates with Raf(340D) to activate Elk-1 but not SRF or c-Jun. The synergistic interaction that we have observed between Raf(340D) and FGD1 in a primary focus formation assay may be the consequence of cooperativity in the activation of transcriptional pathways. c-Jun and Elk-1 are activated by distinct mitogen-activated protein kinase cascades

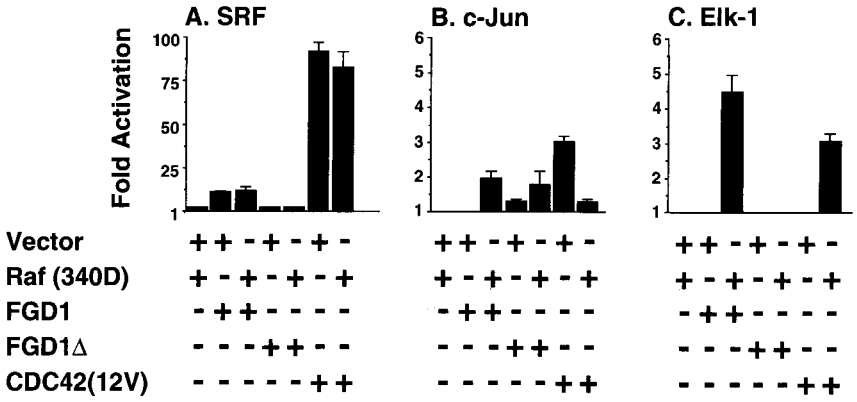

FIG. 4. FGD1 and CDC42(12V) cooperate with Raf to stimulate Elk-1 but not SRF or c-Jun transcriptional activity. NIH 3T3 cells were cotransfected with pAX142 or pZIP-NeoSV(x)1 (500 ng) expression plasmids encoding the indicated proteins along with luciferase gene reporter constructs for SRF transcriptional activity (2.5 μg) (A), c-Jun transcriptional activity (500 ng of Gal-Jun and 2.5 μg of 5×Gal) (B), or Elk-1 transcriptional activity (500 ng of Gal-Elk and 2.5 μg of 5×Gal) (C). Transcriptional assays were performed as described in the legend to Fig. 3. Data shown are representative of three independent experiments performed on duplicate plates. Error bars, standard errors.

(e.g., JNK, p38, and extracellular signal-related kinase [ERK]) that have been implicated in the regulation of transforming pathways in NIH 3T3 cells (reviewed in reference 67). In addition, we have shown previously that the activation of SRF-mediated pathways correlates well with the transforming activity of Dbl family members and, in particular, those that have GEF activity for CDC42 (56). To assess possible contributions of SRF-, c-Jun-, and Elk-1-mediated pathways to FGD1 transforming activity, we examined whether Raf(340D) cooperates with FGD1 to activate these response elements.

Both activated CDC42 and FGD1 cooperated with Raf to activate the Elk-1 responsive element (four- and threefold above additive levels, respectively), but little or no cooperation was observed in the SRF or c-Jun assays (Fig. 4). This suggests that Elk-1-mediated signaling, but not that mediated by SRF or c-Jun, may contribute to FGD1- and CDC42-mediated transforming activity. However, since both FGD1 and CDC42 cooperate with Raf to activate Elk-1, this activation cannot fully account for the qualitative and quantitative differences that we have observed in CDC42 + Raf and FGD1 + Raf focus assays. Even though the cooperation between FGD1 and Raf in the Elk-1 assay is consistently higher than that between CDC42 and Raf, it is unlikely that this marginal difference (four- versus threefold) in activity can fully account for these striking differences. We conclude that the differences in transforming potency that we observed between FGD1 and CDC42 are attributable to the differential activation of additional signaling pathways that we have not yet identified.

FGD1 activates Elk-1, SRF, and c-Jun via CDC42-regulated pathways. Our observation that FGD1 and CDC42 have differing abilities to stimulate transcriptional and transforming events suggests that not all FGD1 activity is mediated by its interactions with CDC42. To explore this possibility further, we wished to determine if FGD1 stimulation of transcription from the Elk-1, c-Jun, and SRF reporters is impaired by coexpression with specific inhibitors of CDC42 function. pAX142-*cdc42*(*17N*) encodes a GTPase-defective dominant-inhibitory mutant of CDC42 (provided by R. Cerione). pyDF30-*WASP-GBD* encodes the GBD of WASP (an effector protein specific for CDC42) and has been shown to specifically inhibit CDC42- and FGD1-mediated cytoskeletal rearrangements (36, 40, 52). Coexpression of FGD1 with either WASP-GBD or CDC42 (17N) markedly inhibits its ability to activate SRF, Elk-1, or c-Jun (Fig. 5). To test for possible toxicity of either WASP-GBD or CDC42(17N), we cotransfected NIH 3T3 cells with pAX142-β-*galactosidase* and either pyDF30-*WASP-GBD* or pAX142-*cdc42*(*17N*). β-Galactosidase activity was equivalent in cells transfected with pyDF30-*WASP-GBD*, with pAX142-*cdc42*(*17N*), or with vector alone (data not shown), thus indicating that WASP-GBD and CDC42(17N) do not kill NIH 3T3

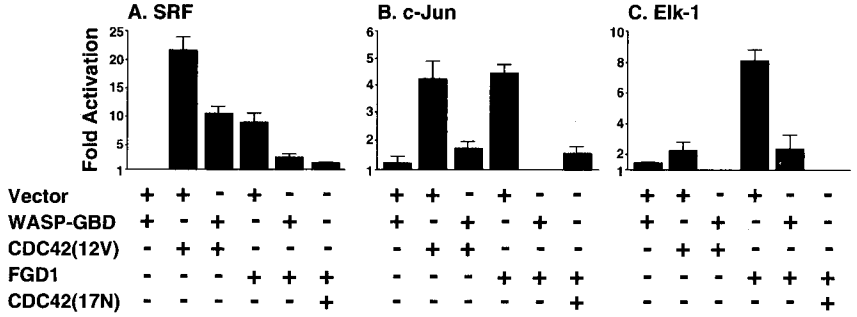

FIG. 5. FGD1 stimulates SRF, Elk-1, and c-Jun activity in a CDC42-dependent manner. NIH 3T3 cells were cotransfected with expression plasmids encoding the indicated proteins (1.5 μg of pAX142, pAX142-myc-*FGD1*, pAX142-*cdc42*(*12V*), or pAX142-*cdc42*(*17N*); 0.25 μg of pyDF30 or pyDF30-*WASP-GBD*) along with luciferase gene reporter constructs for SRF transcriptional activity (2.5 μg) (A), c-Jun transcriptional activity (500 ng of Gal-Jun and 2.5 μg of 5×Gal) (B), or Elk-1 transcriptional activity (500 ng of Gal-Elk and 2.5 μg of 5×Gal) (C). Transcriptional assays were performed as described in the legend to Fig. 3. Data shown are representative of three independent experiments performed on duplicate plates. Error bars, standard error.

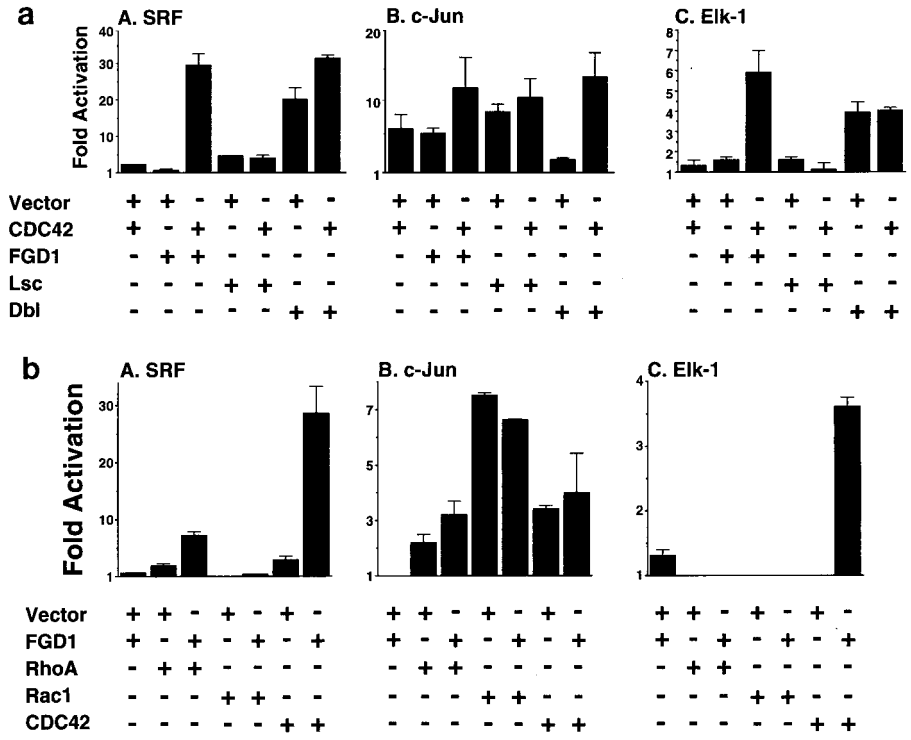

FIG. 6. FGD1 cooperates with RhoA and CDC42 but not Rac1 to stimulate transcriptional response elements. NIH 3T3 cells were cotransfected with pAX142 expression plasmids encoding the indicated proteins (0.5 μg of pAX142, pAX142-*cdc42*, pAX142-*FGD1*, pAX142-*lsc-D7HA*, and pAX142-*Dbl-HA1* [a] or 0.5 μg of pAX142, pAX142-*FGD1*, pAX142-*rhoA*, pAX142-*rac1*, or pAX142-*cdc42* [b]) along with luciferase gene reporter constructs for SRF transcriptional activity (2.5 μg) (A), c-Jun transcriptional activity (500 ng of Gal-Jun and 2.5 μg of 5×Gal) (B), or Elk-1 transcriptional activity (500 ng of Gal-Elk and 2.5 μg of 5×Gal) (C). Transcriptional assays were performed as described in the legend to Fig. 3. Data shown are representative of three independent experiments performed on duplicate plates. Error bars, standard errors.

cells in transient assays and that they do not inhibit expression from the pAX142-encoded EF1-α promoter. Thus, we conclude that WASP-GBD and CDC42(17N) inhibition is specific and that FGD1 activates c-Jun, Elk-1, and SRF via CDC42-regulated pathways.

FGD1 activates Elk-1 and SRF but not c-Jun by direct interaction with CDC42. We and others have shown that Ras GEFs can stimulate the activity of wild-type Ras to activate transcription from Ras-responsive promoter elements (8, 43, 44). To determine if FGD1 regulates transcriptional activation via direct stimulation of CDC42, we measured FGD1 activation of the Elk-1, Jun, and SRF reporters in the presence of wild-type CDC42. To more precisely measure cooperativity due to synergy, we determined the titers of CDC42 and FGD1 DNA used in these transfections to a level (500 ng) at which they exhibited low transcriptional activity when transfected alone (Fig. 6a). Under these transfection conditions, we observed that FGD1 acted synergistically with wild-type CDC42 to cause transcriptional activation of Elk-1 and SRF (3-fold and 10-fold above additive levels, respectively) but not c-Jun (Fig. 6a). Interestingly, a strongly transforming derivative of Dbl that is consistently more active in Elk-1, c-Jun, and SRF assays than FGD1 (Fig. 6a compares 50 ng of Dbl-HA1 to 500 ng of FGD1) failed to synergize with wild-type CDC42 in the Elk-1 assays yet showed weak cooperativity in the c-Jun and SRF assays. This suggests that Dbl family exchange factors with equivalent in vitro substrates may interact with these substrates, in vivo, in a biologically distinct manner. It further suggests that activation of the JNK pathway by FGD1, although clearly CDC42 dependent, may also require FGD1-mediated signaling events that are independent of CDC42. A plasmid construct encoding an activated derivative of Lsc, a Dbl family protein with GEF activity for RhoA, but not CDC42 (14, 60) failed to cooperate with wild-type CDC42 in these signaling assays, thus indicating the specificity of the synergy for CDC42 GEFs.

Finally, we wished to determine if FGD1 could cooperate with Rho family GTPases other than CDC42 in signaling assays. We observed that under conditions under which FGD1 cooperated with CDC42 in SRF and Elk-1 assays, FGD1 did not cooperate with wild-type Rac1 (Fig. 6b). Interestingly, cooperativity was observed between FGD1 and wild-type RhoA in the SRF assay (threefold above additivity) but not in the c-Jun or Elk-1 assays. The cooperativity between FGD1 and RhoA, although less striking than that between FGD1 and CDC42, may reflect weak in vivo exchange activity of FGD1 for RhoA. In support of this we observed that FGD1-mediated stimulation of transcription from the SRF and Elk-1 reporters but not the c-Jun reporter is impaired by coexpression with RhoA(19N), a dominant-inhibitory mutant of the RhoA protein (Fig. 7).

DISCUSSION

Disruption of the *FGD1* locus by translocation or premature truncation has been detected in patients that suffer from FGDY (also referred to as Aarskog-Scott syndrome) (38). Loss of the *FGD1* locus is associated with a characteristic pattern of growth impairment during development of the skeletal system, thus suggesting that FGD1-mediated signaling pathways may play a crucial role in this process (1, 15, 16). FGD1 is expressed during development in the embryonic fi-

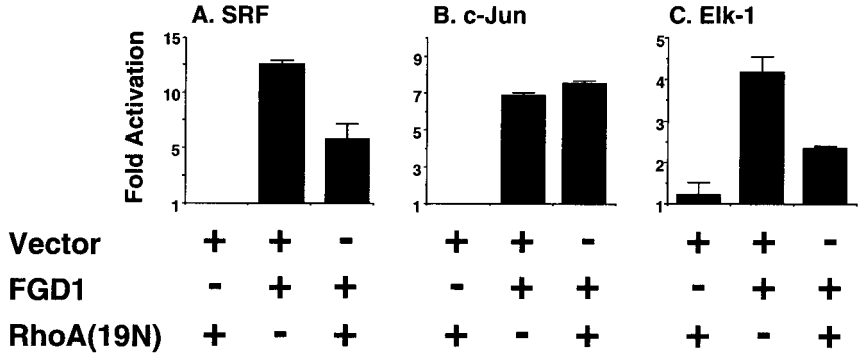

FIG. 7. FGD1 stimulates SRF and Elk-1 but not c-Jun activity in a RhoA-dependent manner. NIH 3T3 cells were cotransfected with expression plasmids encoding the indicated proteins [1.5 μg of pAX142, pAX142-myc-*FGD1*, and pAX142-*rhoA*(*19N*)] along with luciferase gene reporter constructs for SRF transcriptional activity (2.5 μg) (A), c-Jun transcriptional activity (500 ng of Gal-Jun and 2.5 μg of 5×Gal) (B), or Elk-1 transcriptional activity (500 ng of Gal-Elk and 2.5 μg of 5×Gal) (C). Transcriptional assays were performed as described in the legend to Fig. 3. Data shown are representative of three independent experiments performed on duplicate plates. Error bars, standard errors.

broblasts that comprise the early mesenchymal condensations (unpublished observations). Mesenchymal condensations form by a variety of mechanisms, including increased mitotic activity and the aggregation of cells towards a center (17). Our observation that FGD1 expression can disrupt the proliferative properties of NIH 3T3 cells suggests that FGD1 may be a necessary component of growth control pathways that regulate the establishment of these condensations during normal embryonic development. We have shown that expression of the *FGD1* gene product in NIH 3T3 cells is sufficient to cause tumorigenic transformation as well as to activate transcription factors (SRF, Elk-1, and c-Jun) which have demonstrated roles in the regulation of cell growth. In addition, FGD1 synergizes strongly with Raf in transformation assays. The synergy between FGD1 and Raf in transformation assays is associated with an increase in Elk-1 but not SRF or c-Jun activation, thus suggesting that activation of SRF or c-Jun is not necessary for this synergistic interaction.

Whereas the transcriptional activation of c-Jun by FGD1 is consistent with the previous observation that FGD1 is a good stimulator of JNK activity (36, 64), it is unclear by which mechanism FGD1 is stimulating Elk-1 activity. Elk-1 is a target for the mitogen-activated protein kinases ERK (11, 12, 21), JNK (6, 13, 61, 62, 66), and p38 (45, 62). It is unlikely that FGD1 activation of Elk-1 is mediated by ERKs, since we and others have observed that ERK1 and ERK2 are not activated in COS cells by FGD1 (unpublished observations; 36). In addition, we have recently determined that Elk-1 can be activated by many Dbl family members, most of which are not good activators or ERKs (unpublished observations). It also appears that FGD1-mediated Elk-1 activation is not a consequence of JNK activation, since FGD1 cooperates with CDC42 and Raf to activate Elk-1 under conditions where c-Jun (and presumably JNK) activity remains unchanged. The components of the pathway leading from FGD1/CDC42 to Elk-1 activation remain to be elucidated.

Although recent evidence suggests that FGD1 functions exclusively as an activator of CDC42 (36, 64), the transforming activity and signaling profile of FGD1 in the present study were not always consistent with in vivo activation of CDC42. We observed both qualitative and quantitative differences between FGD1 and CDC42(12V) in transformation assays, and both proteins exhibited distinctive patterns of activation of reporter elements. One explanation for these differences is that FGD1 may be utilizing GTPase substrates other than CDC42 to trigger downstream signaling events. In support of this, we observed that FGD1 consistently exhibits cooperativity with wild-type RhoA in SRF reporter assays and that a dominant-inhibitory mutant of RhoA partially blocks signaling by FGD1. Although this suggests that FGD1 may be utilizing RhoA as a substrate in vivo, it is also possible that RhoA is acting downstream of CDC42 to regulate some of the FGD1 signaling activities. We also observed that FGD1 does not cooperate with wild-type CDC42 in an assay for c-Jun activation under conditions under which a second CDC42 GEF (Dbl) does. Thus, although FGD1 activates c-Jun in a CDC42-dependent manner, this activation may be dependent upon additional FGD1-mediated signaling activities. We have shown recently that several Dbl family members have a broader range of in vivo substrate utilization than is indicated by their in vitro activity, and this may also apply to FGD1 (56).

Some of the differences we observed between CDC42 and FGD1 in both signaling and transformation assays may also be attributable to the inability of a constitutively activated mutant to precisely mimic activation of a GTPase by a GEF. The biological consequences of an interaction between an exchange factor and a GTPase are likely to differ for each GEF, and constitutively activated mutants of the GTPase may not always be able to substitute for the GEF-GTPase complex. For example, Dbl family GEFs may sequester their GTPase targets to particular cellular locations and, consequently, regulate differential interactions with specific effectors. Thus, the GEF may need to be present to optimize the stimulation of a particular signaling pathway by the GTPase. If true, this would explain how FGD1 can be a much more efficient activator of Elk-1 than CDC42(12V) yet still clearly activate Elk-1 in a CDC42-dependent manner.

Additionally, it is possible that differences between FGD1 and CDC42 activity may be a phenomenon specific for the cycling-defective CDC42(12V) mutant. Recent observations by Lin et al. with the CDC42(28L) mutant suggest that enhanced GDP/GTP cycling also contributes to downstream signaling (28). However, our preliminary analysis of the CDC42(28L) mutant indicates that it behaves identically to CDC42(12V) in cooperativity focus formation assays with Raf(340D) (unpublished observations), thus suggesting that the observed differences between CDC42 and FGD1 function cannot be simply attributed to a lack of cycling by the CDC42(12V) mutant.

We have also examined the transforming and signaling activities of FGD1Δ, a peptide derived from a naturally occurring

splice variant of FGD1 that harbors a small deletion within its DH domain (37). This deletion removes several residues that are highly conserved in all DH domains and would be predicted to abolish the catalytic activity of FGD1 (59). Consistent with this, we observed that FGD1Δ lacked any measurable transforming or signaling activity when assayed under the same conditions as FGD1. Interestingly, we were unable to select stable cell lines that express detectable levels of the FGD1Δ protein, suggesting that its expression may be toxic or growth inhibitory to NIH 3T3 cells. Since we have also observed that the dominant-negative CDC42(17N) mutant is also growth inhibitory in NIH 3T3 cells (unpublished data), FGD1Δ may form nonproductive interactions with CDC42 and thus may represent a naturally occurring, dominant-inhibitory mutant of FGD1.

In summary, we have presented evidence that expression of the FGD1 protein has profound effects on growth control and nuclear signaling activity in NIH 3T3 cells. Although we have strong in vivo evidence that at least some of these events are mediated by specific interactions between CDC42 and FGD1, it is also clear that FGD1 has functions other than guanine nucleotide exchange on CDC42. FGD1 expression is primarily restricted to fetal and embryonic tissues (38), and recent RNA in situ hybridizations show that FGD1 is predominantly expressed in cell populations from which skeletal precursors arise (unpublished observations). Investigations as to whether FGD1 and CDC42 exhibit a growth-promoting activity in skeletal precursors will be important to pursue. We are currently evaluating whether FGD1 can promote the growth or differentiation of osteoblasts.

ACKNOWLEDGMENTS

We thank Carol Martin and Que Lambert for technical support, Jennifer Parrish for preparation of figures, Marc Symons for the *WASP-GBD* cDNA sequences, and Rick Cerione for the *cdc42*(*WT*), *cdc42*(*17N*), and *cdc42*(*12V*) cDNA sequences.

This work was supported by Public Health Service grants CA42978, CA55008, and CA63071 to C.J.D. from the National Cancer Institute. I.P.W. is a research fellow of the National Cancer Institute of Canada supported with funds provided by the Terry Fox Run.

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