Activation of the Rap1 Guanine Nucleotide Exchange Gene, CalDAG-GEF I, in BXH-2 Murine Myeloid Leukemia

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Here we report the recurrent proviral activation of the Rap1-specific guanine nucleotide exchange factor CalDAG-GEF I (Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13278–13283; Correction (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 318) gene in BXH-2 acute myeloid leukemia. We also show that CalDAG-GEF I encodes two protein isoforms, a full-length isoform (CalDAG-GEF Ia) and a C-terminally truncated isoform (CalDAG-GEF Iib). Expression of the full-length CalDAG-GEF Ia isoform in Rat2 fibroblasts enhances growth in low serum, whereas expression in Swiss 3T3 cells causes morphological transformation and increased saturation density. In FDCP1 myeloid cells, CalDAG-GEF Ia expression increases growth and saturation density in the presence of the diacylglycerol analogs phorbol 12-myristate 13-acetate (PMA), which activates CalDAG-GEF Ia exchange activity. Likewise, in 32Dcl3 myeloblast cells, CalDAG-GEF Ia expression increases cell adherence to fibronectin in response to PMA and calcium ionophore and allows higher saturation densities and prolonged growth on fibronectin-coated plates. These effects were correlated with increased Rap1, but not Ras, protein activation following PMA and calcium ionophore treatment. Our results suggest that Rap1-GTP delivers signals that favor progression through the cell cycle and morphological transformation. The identification of CalDAG-GEF I as a proto-oncogene in BXH-2 acute myeloid leukemia is the first evidence implicating Rap1 signaling in myeloid leukemia.

Retroviral insertional mutagenesis in BXH-2 recombinant inbred mice induces a high incidence of acute myeloid leukemia (AML), and the proviral integration sites in the leukemias provide powerful genetic tags for disease gene identification (2, 3). During the past several years, a number of disease genes have been identified in BXH-2 AMLs by proviral tagging. They include a tumor suppressor gene, Neurofibromatosis type 1 (Nf1); a gene with homology to the lymphoid-restricted type II membrane protein Jaw1, Mv integration site 1 (Mrv1); a gene encoding a hematopoietic cell growth and differentiation factor, myeloblastosis oncogene (Myb); three homeobox genes, homeobox A7 (Hoxa7), homeobox A9 (Hoxa9), and myeloid ectropic viral integration site 1 (Meis1); a zinc finger protein gene, ectropic viral integration site 9 (Evi9); and a novel cytokine receptor (Evi27) (4–12). Importantly, two of these genes, Nf1 and HOXA9, are known human myeloid leukemia disease genes, validating the usefulness of this approach for human disease gene identification (9, 13).

Mutations in the human Nf1 gene are responsible for the cancer predisposition syndrome neurofibromatosis type 1 (13). Nf1 encodes neurofibromin, a GTPase-activating protein (GAP) that is active on the four true Ras proteins (Ha-Ras, K-RasA, K-RasB, and N-Ras) as well as R-Ras (14–16). In BXH2 AMLs, about 15% of the AMLs have proviral insertions in the Nf1 gene (4, 5). These proviral insertions inactivate neurofibromin expression, and no wild type neurofibromin is expressed in these AMLs (5). Loss of neurofibromin in myeloid cells is associated with increased and prolonged Ras activation after cytokine stimulation in chronic myeloid leukemia and in primary cells (17). These results contribute to a large body of evidence implicating aberrant regulation of Ras signaling in myeloid leukemia.

The other 85% of BXH-2 AMLs do not have proviral insertions in the Nf1 gene (4, 5). This has led to the suggestion that these AMLs have insertions in or near other genes involved in Ras regulation and that these insertions activate Ras in the absence of inactivating insertions at Nf1. One class of likely targets are the guanine nucleotide exchange factors (GEF) that catalyze the exchange of GTP for GDP on Ras and consequently turn on Ras. One such GEF is RasGRF (18, 19). RasGRF is one of three guanine exchange factors that have calcium-binding “EF hands” in addition to diacylglycerol (DAG)-binding domains (18–20). It has been speculated that this class of GEFs couple cell surface receptors that signal through Ca2+ and DAG to the Ras pathway (18, 20).

In the studies described here we report the recurrent proviral activation of the calcium- and diacylglycerol-binding guanine nucleotide exchange factor 1 (CalDAG-GEF I) gene in BXH-2 AML. Like RasGRF, CalDAG-GEF I has EF hands and DAG domains. Previous studies, however, have shown that CalDAG-GEF I is not a Ras GEF, rather it appears to be a Rap1
GEF (1). We also show that forced overexpression of CalDAG-GEF I results in transformation of cultured fibroblasts and implicate Rap1 signaling in producing these effects. This is the first evidence implicating Rap1 signaling in myeloid leukemia in human or mouse.

**EXPERIMENTAL PROCEDURES**

Molecular Cloning of CalDAG-GEF I Proviral Insertions—Proviral insertions in the CalDAG-GEF I first intron were discovered as part of a large scale cloning effort that utilized a long template, inverse PCR method, described in detail elsewhere (6). In brief, 5 μg of genomic DNA from individual BXH 2 AMLs was digested with SacII overnight; the enzyme was inactivated by heating at 65 °C for 10 min, and the DNA fragments were ligated in 500-μl reactions, using 5 units of T4 DNA ligase (Stratagene) at 4 °C overnight, to produce circular provirus/cellular DNA templates for PCR amplification. The ligated material was precipitated in ethanol and resuspended in 20 μl of Tris-EDTA (pH 8.0). Two μl of this precipitated material was used as template in a primary PCR in a 50-μl reaction volume containing 20 nmol each of dNTP, 10 pmol each forward and reverse primer, 1× buffer 2, and 2.5 units of enzyme mix in the Expand™ Long Template PCR System (Roche Molecular Biochemicals). Amplification was performed with an Omnigene Hybaid thermocycler programmed as follows: 92 °C for 2 min; 30 cycles for 10 s at 94 °C, 60 °C for 30 s, 68 °C for 10 min; 20 cycles of 92 °C for 10 s, 63 °C for 30 s, 68 °C for 10 min with 20-s auto-extension. The amount of primary PCR product was semi-quantified by 1% agarose gel electrophoresis and 0.01 to 1 μl of the primary PCR product was used as template in a secondary PCR under the same conditions except that the secondary primers were used. The secondary PCR product was separated on a 1% agarose gel, purified using the Geneclean II kit (Bio 101), and directly cloned using CloneAmp™ pAMP1 System (Life Technologies, Inc.) according to supplied protocol. These particular clones were obtained using primer pairs designed to amplify proviruses located 3′ of genomic SacII sites. The primers used in the primary PCR are as follows: 5′-ECOF1 (5′-GGGTCCAGATGACCTT-3′) and 5′-ECO R4 (5′-GGGCTGCTACTCAGTGGACCTT-3′) and 5′-ECO F3 (5′-GGCGCCATCTCCGGATCATG-3′) and 5′-ECO R2-dUMP (5′-CTCTGTCGCCATCTCCGTCAGA-3′). The cloned PCR products were sequenced using the PRISM™ BigDye™ Cycle Sequencing Kit (PerkinElmer Life Sciences) on an ABI model 373A DNA Sequencer (Applied Biosystems). SP6 and T7 sequencing primers were purchased on an ABI model 373A DNA Sequencer (PerkinElmer Life Sciences) on an ABI model 373A DNA Sequencer (Applied Biosystems). SP6 and T7 sequencing primers were purchased from Life Technologies, Inc.

**Southern Blot Analysis**—Isolation and analysis of BXH2 genomic DNA and poly(A+) RNA by Southern and Northern blotting, was performed using random primed [32P]dCTP-labeled probe (Roche Molecular Biochemicals) as described in detail elsewhere (6). In brief, 5 μg of DNA was loaded onto SDS-polyacrylamide gels, run, and transferred to nitrocellulose as described (24). SDS-polyacrylamide gels, run, and transferred to nitrocellulose as described (24). The probes used in these studies were the pcDNA3/V5/His-TOPO vector from Invitrogen. This vector added a Myc epitope tag to the C-terminal end of the CalDAG-GEF Ib protein. Both the CalDAG-GEF IA and CalDAG-GEF Ib expression constructs were sequenced for errors. Both expression constructs were used in an in vitro transcription/translation experiment (Promega) to confirm that they produced an ~70-kDa V5-tagged protein for CalDAG-GEF IA and 19-kDa Myc-tagged protein for CalDAG-GEF Ib. The CalDAG-GEF IA insert was subcloned into the MSCV2.1 empty vector and electroproporated into the PT67 amphotropic packaging cell line (CLONTECH). The electroproporated PT67 cells were selected in 400 μg/ml G418 in 10% fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), and penicillin/streptomycin (P/S). Resistant colonies were pooled and replated at 1–2 × 106 cells/ml. Viral supernatants were collected from individual cultures when the cells were harvested 24 h after cells reached confluence and stored at ~70 °C.

**Cell Culture Assays**—The following cell types were transduced with MSCV-CalDAG-GEF IA or MSCV2.1 empty vector virus and selected in G418: Rat2, Swiss 3T3, FDCP1, and 32Dcl3G418. All lines were obtained from the ATCC, apart from 32Dcl3Gr, which was obtained from D. Askew (University of Cincinnati). Rat2 and Swiss 3T3 were grown in 10% FBS/DMEM supplemented with P/S and selected in 400 μg/ml G418. FDCP1 and 32Dcl3G418 were grown in 20% WEHI-3 cell line conditioned medium in 10% FBS/DMEM supplemented with 10% NCTC-109, P/S, Hepes buffer (pH 7.4), 10 units/ml insulin, glucose, sodium pyruvate, nonessential amino acids, and 10−3 M β-mercaptoethanol. All media and supplements were from Life Technologies, Inc., except insulin and P/S (BioWhittaker). Cells were plated in 1 ml of the primary PCR product was used as template in a secondary PCR under the same conditions except that the secondary primers were used. The secondary PCR product was separated on a 1% agarose gel, purified using the Geneclean II kit (Bio 101), and directly cloned using CloneAmp™ pAMP1 System (Life Technologies, Inc.) according to supplied protocol. These particular clones were obtained using primer pairs designed to amplify proviruses located 3′ of genomic SacII sites. The primers used in the primary PCR are as follows: 5′-ECOF1 (5′-GGGTCCAGATGACCTT-3′) and 5′-ECO R4 (5′-GGGCTGCTACTCAGTGGACCTT-3′) and 5′-ECO F3 (5′-GGCGCCATCTCCGGATCATG-3′) and 5′-ECO R2-dUMP (5′-CTCTGTCGCCATCTCCGTCAGA-3′). The cloned PCR products were sequenced using the PRISM™ BigDye™ Cycle Sequencing Kit (PerkinElmer Life Sciences) on an ABI model 373A DNA Sequencer (Applied Biosystems). SP6 and T7 sequencing primers were purchased from Life Technologies, Inc.

**Southern Blot Analysis**—Stably or transiently transfected cells were lysed in RIPA buffer (10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonident P-40, 0.5% deoxycholate, 0.1% SDS) at a concentration of 10 × 106 cells/ml at 4 °C. The filters were hybridized with radiolabeled probes using standard procedures. The filters were washed and quantitated by autoradiography. The blots were hybridized with anti-tubulin or anti-V5 antibody (Invitrogen) according to manufacturer’s instructions and visualized with the ECL chemiluminescence kit (Amer sham Pharmacia Biotech).

**Expression Constructs, Electroporation, and Retroviral Vectors**—The CalDAG-GEF IA and CalDAG-GEF Ib open reading frames were cloned from a mouse bone marrow cDNA library using the HIFI polymerase chain reaction kit from Roche Molecular Biochemicals. Primer sequences used to amplify full-length CalDAG-GEF I are forward 5′-ACCGCGACGGATGACGA-3′ and reverse 5′-GGTTAGTACCATCAAACACCTCGCTCCTC-3′. PCR products were gel-purified and cloned into the pcdNA3.1/V5/HisTOPO vector from Invitrogen. This vector added a V5 epitope tag to the C-terminal end of the CalDAG-GEF IA protein. Primer sequences used to amplify full-length CalDAG-GEF Ib were forward 5′-CTGAGTTGCCTGGGATCATG-3′ and reverse 5′-CTCAAAAGGATCATGTTGATGACCTG-3′. These primers added a XhoI site and a XmnI site to the 5′ and 3′ ends of the PCR product, respectively. PCR products were gel-purified, digested with XhoI and XmnI, and then cloned into the XhoI and XmnI sites of the pcdNA3.1/V5/Myc-His(+) from Invitrogen. This vector added a Myc epitope tag to the C-terminal end of the CalDAG-GEF Ib protein.
Ral GDS fused to glutathione S-transferase was used to isolate Rap GTP from one-half of the sample on glutathione-Sepharose beads (25). The GTP was then eluted from Rap and measured as described above.

To measure the sum of GTP and GDP bound to Rap in the other half of the sample, extracts were incubated in the absence of magnesium with 10 μM GTP to convert Rap-GDP to Rap-GTP. Excess free GTP was removed by a temperature-dependent phase extraction since, as part of the procedure for measuring Rap1 activation, cells were extracted in 0.92% Triton X-114, 0.08% Triton X-45; an aqueous phase containing the free GTP and a detergent phase containing the Rap1 were generated by warming the samples to 15 °C for 2 min. Rap-GTP, which now represented the sum of Rap-GTP plus Rap-GDP, was then isolated by binding to the Rap binding domain glutathione S-transferase fusion protein, and the GTP was eluted and measured as described above.

RESULTS

Proviral Integration at CalDAG-GEF I in BXH-2 AML Leads to Increased CalDAG-GEF I Expression—Proviral insertions within the first intron of the CalDAG-GEF I gene were identified as part of a large screen to discover proviral insertions that are located near CpG islands (6). Sequence analysis of two of the clones showed that they were located in the first intron of the mouse homolog of the human CDC25-like gene (Fig. 1A) also called CalDAG-GEF I (1). Northern blot analysis of one of the leukemias (number 22) showed that CalDAG-GEF I expression was elevated compared with other BXH-2 leukemias (numbers 13, 29, 106, 117, and 132) that did not contain viral integrations at CalDAG-GEF I (Fig. 1B). These results show that viral integration at CalDAG-GEF I leads to increased CalDAG-GEF I expression.

Two CalDAG-GEF I-hybridizing transcripts were detected in all BXH-2 leukemias tested. This was surprising because the shorter transcript has not been reported in humans (1). Interestingly, both transcripts were up-regulated by viral integration at CalDAG-GEF I (Fig. 1B). This prompted us to examine normal mouse tissues for the shorter CalDAG-GEF I transcript. As shown in Fig. 2, both CalDAG-GEF I transcripts are expressed in normal mouse tissues. Expression was highest in brain, heart, and lung followed by spleen, liver and kidney. Expression was not detected in skeletal muscle. Both transcripts are also expressed in the embryo with the highest expression found between 15 and 17 days of development. Subsequently, we confirmed that the shorter transcript is also expressed in humans (data not shown). The shorter transcript hybridized to probes composed of CalDAG-GEF I exons 1–5 but not with probes from farther downstream (data not shown). In

![FIG. 1.](image1)

A, proviral insertions in the first intron of the CalDAG-GEF I gene. Exons are shown as black boxes, and the position and orientation of the proviruses at CalDAG-GEF I are indicated by an arrow. B, Northern blot analysis of BXH-2 AMLs with proviral insertions at CalDAG-GEF I. BXH-2 poly(A)^+ RNAs are shown hybridized with a CalDAG-GEF Ib cDNA probe. The size (in kilobase pairs (kb)) and position of migration of RNA markers are indicated to the left. Tumor 22 has a proviral insertion in the first intron of the CalDAG-GEF I gene. Below the blot are shown the Gapdh-normalized CalDAG-GEF I transcript levels as determined using PhosphorImager analysis.

![FIG. 2.](image2)

Expression of CalDAG-GEF I mRNA in adult tissues and the embryo. Poly(A)^+ RNA, isolated from day 7, 11, 15, and 17 normal mouse embryos or normal adult heart (Hr), brain (Br), spleen (Sp), lung (Lg), liver (L), skeletal muscle (Sm), kidney (Kd), and testis (Ts), was hybridized with a CalDAG-GEF Ib cDNA probe. CalDAG-GEF Ia and CalDAG-GEF Ib transcripts are indicated.
fact, the size of the shorter transcript is approximately what
would be expected for an mRNA that was polyadenylated
shortly after the fifth exon. Quantitation by PhosphorImager
analysis of normal tissue Northern blots, hybridized with a
probe containing exons 1–5, showed that the long and short
transcripts are usually present at a roughly 1:1 ratio, although
in some tissues more long form is expressed (Fig. 2).

Alternate Polyadenylation Predicts the Expression of a Full-
length (CalDAG-GEF Ia) and a C-terminal Truncated
(CalDAG-GEF Ib) Protein—Sequence analysis of the human
CalDAG-GEF I genomic locus revealed a putative alternate
polyadenylation site that was located just after the splice donor
site within intron 5 (Fig. 3). This polyadenylation site is also
present in the mouse. Examination of EST data bases revealed
a number of mouse and human ESTs representing cDNA clones
in which polyadenylation apparently occurred at this site
rather than at the end of the last
CalDAG-GEF I
exon. These
cDNA clones contain CalDAG-GEF I sequences from exon 5,
which then read into the fifth intron and are polyadenylated
after a consensus ATAATA site. Polyadenylation at this site
would produce a transcript of ~0.6 kilobase pairs.

The short CalDAG-GEF Ib transcript is predicted to encode
a truncated protein. CalDAG-GEF Ib would include the Ras
exchanger motif (REM) domain, amino acid sequences between
the REM domain and CDC25 domain (i.e. the catalytic core
domain similar to that from the yeast CDC25 gene), and 17
(mouse) or 20 (human) amino acids encoded by intron 5 (Fig. 3).
Comparison between the human and mouse CalDAG-GEF I
genomic sequences showed conservation in the region sur-
rounding the alternate polyadenylation site in the fifth intron
and indeed the whole fifth intron (72% identity). In contrast,
other intron sequences showed much less conservation (e.g.
fourth intron, 51% identity). These data indicate that the
CalDAG-GEF I locus produces two transcripts through alter-
nate polyadenylation, which encode a full-length protein,
CalDAG-GEF Ia, and a truncated protein, CalDAG-GEF Ib.

Effects of CalDAG-GEF Ia Overexpression in Fibroblasts and
Myeloid Cells—Both CalDAG-GEF I transcripts were cloned
into mammalian expression vectors through PCR amplification
of lymph node cDNA. Primers were designed for both tran-
scripts to allow the PCR fragments to be cloned in-frame into
an expression vector and to add an epitope tag to the 3
end of each cDNA. This resulted in a Cdgla clone tagged with a V5
epitope and a CalDAG-GEF Ib clone tagged with a Myc epitope.
The cytomegalovirus promoter was used to drive both con-
structs. To determine whether the expression vectors func-
tioned as expected, we transiently transfected each vector into
HeLa cells and then followed their expression using antibodies
to the epitope tag. Surprisingly, we were unable to detect
CalDAG-GEF Ib protein expression by Western analysis. This
contrasts with in vitro transcription/translation studies where
we were able to detect Cgd1b epitope-tagged protein (data not
shown). We have also failed to generate stable CalDAG-GEF
Ib-expressing transfectants. These results suggest that the
CalDAG-GEF Ib protein is toxic, unstable, or produced at low
levels.

To test for possible transforming effects of the CalDAG-GEF
Ia protein, an MSCV2.1-based expression vector for CalDAG-
GEF Ia was constructed (MSCV-Caldag-GEF Ia) and tested
for its ability to express V5 epitope-tagged CalDAG-GEF Ia
protein in FDCP1, Rat2, and 32Dcl3Gr cells. All MSCV-
CalDAG-GEF Ia vector transduced cells expressed readily de-
tectable protein (Fig. 4). The Rat2 fibroblast cell line was sub-
sequently transduced with CalDAG-GEF Ia-expressing virus
or empty vector virus pools and selected in G418. Stable Rat2
transductants were obtained, and pools of greater than 50
colonies of each were assayed for growth in soft agar and
growth rates in 0.5 or 5% FBS, with or without added PMA, a
diacylglycerol analog, were measured. Neither population con-
tained cells capable of forming large colonies in soft agar (data
not shown). The number of colonies formed in liquid culture in 0.5% FBS/DMEM was significantly higher for MSCV-CalDAG-GEF Ia transductants than for the empty vector transductants both with and without added PMA (Fig. 5A). At higher serum concentrations, both populations gave rise to a similar number of colonies.

In contrast to Rat2 cells, Swiss 3T3 cells transduced with MSCV-CalDAG-GEF Ia formed multiple, transformed foci upon reaching confluence after G418 selection (Fig. 5B). These foci appeared as areas of more spindle-shaped cells, growing at high density and in multiple layers. The size of these foci increased with time in culture. Upon replating, many secondary foci were present in cultures transduced with the MSCV-CalDAG-GEF Ia vector. These transformed foci were more apparent in 10% FBS than in 5% calf serum (CS) medium. The CalDAG-GEF Ia-transduced Swiss 3T3 cells also reached a higher saturation density than did empty vector transductants (Fig. 5C).

The MSCV-CalDAG-GEF Ia and empty vector retroviruses were also used to transduce cells from the myeloid cell lines 32Dcl3Gr and FDCF1 to test the effects of CalDAG-GEF Ia overexpression on myeloid cells. The growth and viability of 32Dcl3Gr and FDCF1 cells are dependent on the presence of interleukin-3 (IL-3) in the culture medium. If IL-3 is removed from the culture medium, these cells will die by apoptosis. Previous studies have suggested that Rap1 plays a role in regulating integrin-mediated cell adhesion to fibronectin (26–28) and therefore fibronectin was included in some of these experiments.

The addition of PMA (50 ng/ml, in the absence of IL-3, caused a slight delay in apoptosis in MSCV-CalDAG-GEF Ia-transduced but not control empty vector-transduced 32Dcl3Gr cells, which was most apparent in fibronectin-coated plates (data not shown). PMA and calcium ionophore-treated MSCV-CalDAG-GEF Ia-transduced 32Dcl3Gr cells displayed a substantial increase in adherence to fibronectin compared with controls (Fig. 6A). FDCF1 cells expressing CalDAG-GEF Ia at high levels did not become IL-3-independent but showed a subtle increase in growth rate and saturation density compared with empty vector transductants (Fig. 6B). However, the difference in growth rate and saturation density was evident only in the presence of 10 or 50 ng/ml PMA. PMA activates the guanine nucleotide exchange activity of CalDAG-GEF I (1).

Rap1, but Not Ras, Activation in Cells Expressing CalDAG-GEF Ia at High Levels—To determine whether enforced expression of CalDAG-GEF Ia in cells resulted in increased endogenous Rap1, activation of Rap1 in transfected cells, in myeloid cells, was measured. Endogenous Rap-GDP and Rap-GTP in empty vector or MSCV-CalDAG-GEF Ia-transduced populations of 32Dcl3Gr cells were measured. These cells werestarved of serum and IL-3 for 10 h and stimulated with calcium ionophore and PMA to activate CalDAG-GEF Ia for 15 min. CalDAG-GEF Ia-transduced cells showed an increase in induced Rap-GTP levels at 15 min compared with control cells (Fig. 7). The levels of Ras-GTP did not vary significantly between CalDAG-GEF Ia and parental 32Dcl3Gr cells during this time (Fig. 7). This is consistent with other reports showing that CalDAG-GEF I overexpression does not affect Ras-GTP levels in transiently transfected cells (1). Thus, CalDAG-GEF Ia expression at high levels can influence the level of endogenous Rap1 that becomes activated in response to stimulation.

As has been reported in HL60 cells (25), Rap1 protein in 32Dcl3Gr cells shows a relatively high basal state of activation compared with Ras (Fig. 7). This has been suggested to be due to the presence of a threonine at position 61 in Rap1, which decreases its intrinsic GTPase activity (25).

DISCUSSION

Proviral Activation of a Rap1 GEF Gene—A screen of somatic murine leukemia virus integration sites in BHX-2 AML has identified a GEF gene, CalDAG-GEF I, that is a target for murine leukemia virus integration in BHX2 AMLs. Proviral integration at CalDAG-GEF I leads to increased CalDAG-GEF I expression suggesting that CalDAG-GEF I is a proto-oncogene. CalDAG-GEF I is the second GEF to be implicated as a myeloid leukemia disease gene in humans or mouse. The NUP98 gene is fused in frame, by translocation, to the broadly active GEF gene smgD6S in some human myeloid leukemias, the result of which may be an increase in GTPase activation levels (29).

CalDAG-GEF I is closely related to another GEF, RasGRP (1, 18, 19, 20). RasGRP has been shown to have GEF activity for Ha-Ras but little activity for other Ras superfamily members such as Rap, Rho, or Rac (1, 18, 19). In contrast, CalDAG-GEF I shows little GEF activity for Ha-Ras but very high activity for Rap1 (1). More recently, RasGRP, CalDAG-GEF I, and a newly identified GEF, CalDAG-GEF III, have also been shown to have exchange activity for R-Ras and TC21 GTPases (16, 30). Furthermore, another recent paper (31) suggests that CalDAG-GEF I can cause exchange on N-Ras and K-Ras but not Ha-Ras proteins in cells that are chronically stimulated with PMA or high serum. Thus, activation of Rap1, Ha-Ras, N-Ras, R-Ras, and TC21 GTPases could potentially mediate the oncogenic effects of CalDAG-GEF I.

Small G Protein Signaling and Cancer—There is abundant genetic evidence from studies of primary cancers, cancer models, and transformation systems for a central role for the so-called "true" Ras genes (HRAS, NRAS, and KRAS) in cancer development (32, 33). Much less is known, however, about the role of other small G proteins of the Ras-like subfamily in oncogenesis. Rap1 was initially identified in a screen for genes that can suppress the transformed phenotype of K-Ras-transformed fibroblasts (34). This would suggest that Rap1 signals suppress rather than promote oncogenesis. Later publications (35–37) showed that the Rap1 effect domain, being virtually identical to that of Ras, can bind to many Ras effectors without activating them. These data have led to the hypothesis that Rap1 serves as a Ras antagonist by sequestering Ras effectors. More recent work (38), however, has shown that Ras activity is not inhibited by endogenous Rap1 activation, suggesting that Rap1 has distinct biological functions, apart from the inhibition of Ras.

Support for this hypothesis has come from studies of the TSC2 tumor suppressor gene. TSC2 encodes tuberin, which has Rap1 GAP activity (39). In addition, two common human gliomas, astrocytoma and ependymoma, have been shown to have increased Rap1 expression or reduced/absent tuberin expression in 50–60% of the tumors examined (40). In Swiss 3T3 cells, Rap1-GTP can induce DNA synthesis and a transformed morphology (41). Expression of wild type Rap1 at high levels in Swiss 3T3 cells causes morphological transformation and tumorigenicity in nude mice (42). This phenotype is similar to our...
observations with CalDAG-GEF Ia, which causes morphologi-
cal transformation of Swiss 3T3 cells but not NIH 3T3 or Rat2
cells.

Our data suggest that signaling via CalDAG-GEF Ia in my-
eloid cells can promote cellular proliferation and increase ad-
herence. This is consistent with other published data, which
also suggest that Rap1 signaling regulates adherence. For ex-
ample, overexpression of the Rap1 GAP, SPA-1 in 32Dcl3 cells
leads to a block in Rap1 activation and adherence during gran-
ulocyte-colony stimulating factor induced differentiation (28).
Rap1 also seems to mediate adhesion induced by CD31 activa-
tion in lymphoid cells (27) and is a major LFA1 activator,
permitting adhesion to fibronectin (26). Our results are consist-
ent with a role for Rap1 activation in adherence to fibronectin
in myeloid cells. It could be imagined that adhesion to fibronec-
tin gives an AML clone a selective advantage, either by sup-
pression of apoptosis via the so-called “outside-in” integrin
signal or by permitting the clone to colonize extramedullary
sites or extravasate more readily.

Other data indirectly implicate Rap1 signaling in cell cycle
control and proliferation. Expression of the Rap1 GAP, tuberin,
regulates the abundance and subcellular distribution of p27
and cyclin D1 proteins in fibroblasts (43). Our data suggest
that overexpression of CalDAG-GEF Ia can cause inappropri-
ate cell division in Rat2 cells at low serum. This may be anal-
ogous to the effects seen after tuberin depletion in other fibro-
blast cell lines (43).

It is also possible that R-Ras and/or TC21 mediate the effects
of CalDAG-GEF Ia overexpression. Indeed, TC21 (43–45) and
R-Ras (46, 47) can cause malignant transformation of rodent
fibroblasts. TC21 has been shown to be overexpressed in breast
carcinoma and activated by amino acid substitution or inser-
tional mutation in some breast and ovarian cancer cell lines
(48–50). Furthermore, activated R-Ras can suppress apoptosis
and stimulate adhesion in 32Dcl3 cells (51). Finally, recent
data suggest that CalDAG-GEF I is produced as both the form
we have identified and a longer form, referred to as RasGRP2.
Both forms were shown to be capable of GEF
activity in cells for N-Ras or K-Ras. However, the shorter
nonfatty acid modified form of CalDAG-GEF I was only capable
of N- and K-Ras GEF activity after chronic PMA treatment or
growth in high serum, when it becomes localized to the plasma
membrane. However, it is not clear that the longer form of this
protein, called RasGRP2, is actually conserved in the mouse.
We have looked for mouse EST clones, analogous to the human
RasGRP2 isoform, but could find none. Furthermore, the alter-
nate exon that encodes most of the additional N-terminal

FIG. 5. Activity of CalDAG-GEF Ia
in Rat2 and Swiss 3T3 cells. A, colony
formation at low serum in Rat2 cells. The
number of colonies present after seeding
4 × 10^5 cells per plate of MSCV2.1-trans-
duced Rat 2 cells (white bars) or MSCV-
CalDAG-GEF Ia-transduced Rat2 cells
(black bars) is indicated. Error bars indi-
cate standard deviations. B, transformed
foci in CalDAG-GEF Ia-transduced Swiss
3T3 cells. Photomicrograph of parental
Swiss 3T3 cells, MSCV2.1-transduced
Swiss 3T3 cells (MSCV2.1), and of four
different transformed foci of MSCV-
CalDAG-GEF Ia-transduced Swiss 3T3
cells (MSCV-CalDAG-GEF Ia). C, pri-
mary and secondary foci and saturation
density in Swiss 3T3. The number of
Swiss 3T3 cells per 10-cm dish at conflu-
ency is shown for MSCV2.1-transduced
cells (white bars) and for MSCV-CalDAG-
GEF Ia-transduced Swiss 3T3 cells (black
bars). The experiment was performed in
10% FBS or 5% CS. In addition, the aver-
age number of transformed foci of paren-
tal Swiss 3T3, MSCV2.1-transduced,
or MSCV-CalDAG-GEF Ia-transduced
Swiss 3T3 cells is shown. Primary foci are
the number that appeared after G418 se-
lection and growth to confluency. Seco-
dary foci are the number that appeared
after replating and growth to confluency.
Error bars indicate standard deviations.
Error bars indicate standard deviations.

(a) The cell number for MSCV2.1-transduced FDCP1 cells (black bars) or MSCV-CalDAG-GEF Ia-transduced FDCP1 cells (white bars) are shown. Cells were initially plated in normal growth media with IL-3 at 0, 10, or 50 μg/ml PMA, and the cell number was determined every day for 4 days. Error bars indicate standard deviations.

(b) The percentage of total Rap1 GTP bound to TGF or total Ras bound to GTP is indicated for 32Dc3Gr cells transduced with MSCV2.1-empty vector or MSCV-CalDAG-GEF Ia viruses. These values are from cells at time 0 (white bars) and 15 min after stimulation (black bars) of IL-3 and serum-starved cells with calcium ionophore and PMA. The average of an experiment done in triplicate is shown.

![Graph showing the activity of CalDAG-GEF Ia in FDCP1 and 32Dc3Gr cells](image)

**Fig. 6. Activity of CalDAG-GEF Ia in FDCP1 and 32Dc3Gr cells.** A, appearance of adherent cells in PMA plus calcium ionophore (white boxes; PMA + Ca) and IL-3 plus PMA plus calcium ionophore (black boxes; IL-3 + PMA + Ca)-treated 32Dc3Gr cells. The average number of cells per field (at × magnification) remaining on the tissue culture dish 2 h after replating MSCV2.1-empty vector transductants or MSCV-CalDAG-GEF Ia transductants and washing in PBS is shown. Standard deviations are indicated. B, fold increase at saturation density of FDCP1 cells growing in IL-3. The maximal fold increases in cell number for MSCV2.1-transduced FDCP1 cells (white boxes) or MSCV-CalDAG-GEF Ia-transduced FDCP1 cells (black boxes) are shown. Cells were initially plated in normal growth media with IL-3 at 0, 10, or 50 μg/ml PMA, and the cell number was determined every day for 4 days. Error bars indicate standard deviations.

Amino acids in RasGRP2 is not well conserved at the mouse CalDAG-GEF I locus (data not shown). In addition, we have not detected increased Ras activation in CalDAG-GEF I overexpressing 32Dc3 or FDCP1 cells. Nevertheless, it is conceivable, and seems appealing, to consider that the combined activation of multiple small GTPases by CalDAG-GEF I is responsible for its oncogenicity.

It is at present unclear which of the signaling pathways downstream of Rap1, TC21, Ha-, N- or R-Ras activation can be linked to apoptosis suppression, adherence, or hyperproliferation seen in CalDAG-GEF Ia-overexpressing cells. Rap1 signaling has been shown to activate the mitogen-activated protein kinase pathway independently of Ras signaling via B-Raf (52, 53). In addition, Rap1-GTP, like Ras, can bind to the Raf guanine exchange factors RafGDS and Rgl1 (35, 36). The function of Raf-GTP is not known, but Raf dominant-negative mutants block R-Ras-induced adhesion to fibronectin in 32D cells (51). Raf is also thought to be downstream of Ras signaling and forms a required component of the Ras transformation response (54, 55). TC21 and R-Ras have been shown to activate the stress-activated protein kinases, p38 and JNK, as well as phosphatidylinositol 3-kinase (44–47). Further work will be required to determine which of these pathways is important for CalDAG-GEF I-induced leukemia. Furthermore, the CalDAG-GEF II (RasGRP1) Ras GEF gene is found at a common site of proviral insertion and may be activated (6), and the Ras GAP NF1 gene is inactivated in BXH2 AML (4, 5). Therefore, it will be important to determine whether and where Rap1 and N-, K-, or Ha-Ras signalings overlap. Indeed, a lot of data have accumulated showing that small G proteins can cooperate in cell growth control (56) or must act in concert for cellular transformation to occur (37, 51, 57–60).

**Functional Role of a Truncated GEF—CalDAG-GEF Ib, the** truncated form of CalDAG-GEF Ia, is unique in that no other GEFs identified thus far have a truncated form. The simplest model to explain the function of CalDAG-GEF Ib is that it acts as a dominant-negative form of CalDAG-GEF Ia, serving to modulate its activity. The only identified domain within CalDAG-GEF Ib is the REM domain. A REM-like domain is found N-terminal to the core catalytic CDC25-like domain of many GEF proteins. Although the function of the REM domain is not entirely clear, an intact REM domain is required for the transforming effects of RasGRP overexpression in fibroblasts (19). The CDC25-like core catalytic domain of CDC25Mm will catalyze the exchange of GDP for GTP on purified Ras protein, but the inclusion of the REM domain increases the efficiency of this reaction (61).

The crystallization and structural determination of Ha-Ras protein complexed with a fragment of the SOS protein lends some insight into the role of the REM domain (62). The REM domain of SOS contains three α-helices. The first two of the helices interact with, and may stabilize, a portion of the CDC25-like domain (62). The third α-helix and the region of the protein between the REM domain and the CDC25-like domain interact with neither the core catalytic CDC25-like domain nor Ha-Ras. This portion of SOS may thus interact with other proteins. The amino acid sequence of the region between the REM and CDC25-like domains is not conserved between SOS and CalDAG-GEF I. The conservation of a short form of CalDAG-GEF I, CalDAG-GEF Ib, in both mouse and human that includes the REM domain and most of the amino acid sequence between the REM domain and the CDC25-like domain, implies that this region of the protein may have an unidentified and unique regulatory function. For example, CalDAG-GEF Ib may regulate CalDAG-GEF Ia activity by...
interacting with and titrating a protein(s) that also binds to and regulates CalDAG-GEF Iα and perhaps other GFs.

CalDAG-GEF I in Other Malignancies—CalDAG-GEF I maps to human chromosome 11q13 (20). The 11q13 region has been implicated in a variety of malignancies including breast adenocarcinoma, bladder carcinoma, gastric cancer, B cell follicular lymphoma, and oral squamous cell carcinoma (63, 64). Both translocations and gene amplifications have been observed in these cases. It is widely believed that activation of the cyclin D1 gene (CCND1) or fibroblast growth factor superfamily genes, such as FGF and HIS, are the targets for genomic alterations in this region (64). However, our identification of CalDAG-GEF I as a proto-oncogene in BHX-2 AML suggests that its activation may also be selected in some of these cases. Regarding these tumors, it seems possible that selection is occurring due to effects on multiple genes. Rap activity has been implicated in changes in the adhesion and migratory capacity of cells in culture (26–28). Thus, it seems plausible that CalDAG-GEF I gene activation could play a role in the metastatic behavior of these carcinomas.

REFERENCES

1. Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, J. E., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) J. Virol. 69, 3095–3102
2. Nakamura, T., Yamauchi, Y., Saiki, Y., Moriyama, M., Largaespada, D. A., Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) J. Virol. 69, 3095–3102
3. Nakamura, T., Yamauchi, Y., Saiki, Y., Moriyama, M., Largaespada, D. A., Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) J. Virol. 69, 3095–3102
4. Nakamura, T., Yamauchi, Y., Saiki, Y., Moriyama, M., Largaespada, D. A., Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) J. Virol. 69, 3095–3102
5. Nakamura, T., Yamauchi, Y., Saiki, Y., Moriyama, M., Largaespada, D. A., Kawasaki, H., Springett, G. M., Toki, S., Canales, J. J., Harlan, P., Blumenstiel, J. P., Chen, E. J., Bany, I. A., Mochizuki, N., Ashbacher, A., Matsuda, M., Housman, D. E., and Graybiel, A. M. (1998) J. Virol. 69, 3095–3102