Yeast two-hybrid techniques were used to identify possible effectors for the heterotrimeric G protein \( G_\alpha \), in human bone marrow cells. Eya2, a human homologue of the Drosophila Eya transcription co-activator, was identified. Eya2 interacts with activated \( G_\alpha \) and at least one other member of the \( G_\alpha \) family, \( G_{\alpha_\beta} \). Interactions were confirmed in mammalian two-hybrid and glutathione S-transferase fusion protein pull-down assays. Regions of Eya2-mediating interaction were mapped to the C-terminal Eya consensus domain. Eya2 is an intrinsically cytosolic protein that is translocated to the nucleus by members of the Six homeodomain-containing family of proteins. Activated \( G_\alpha \) and \( G_{\alpha_\beta} \) prevent Eya2 translocation and inhibit Six/Eya2-mediated activation of a reporter gene controlled through the MEF3/TATA promoter. Although G proteins are known to regulate the activity of numerous transcription factors, this regulation is normally achieved indirectly via one or more intermediates. We show here a novel functional regulation of a co-activator directly by G protein subunits.

The ability of a cell to respond to external cues is dependent on a large array of receptors and downstream signaling pathways. Heterotrimeric G proteins contribute to these pathways by helping to conduct the flow of information from agonist-activated heptahelical receptors to a variety of intracellular targets (1–3). G proteins have traditionally been studied within the context of second messenger regulation, where adenylyl cyclases, phospholipases, phosphodiesterases, and ion channels assume prominent roles. A substantial amount of attention has been focused more recently on forms of transduction involving protein recruitment apart from second messenger events, for example interacting with regulators of G protein signaling (RGS \( \beta \) molecules), which hasten deactivation of \( G_\alpha \) but can coordinate other proteins (e.g. RhO (10, 11)). \( G_\alpha \) and \( G_{\alpha_\beta} \) interact with Rap1 GTPase-activating protein (12, 13), and one or more \( G_\alpha \) family members interact with G protein-regulated inducers of neurite outgrowth (14) and a Ca\(^{2+}\)-binding protein (15).

G proteins regulate the activity of numerous transcription factors typically through second messengers and quite often through other intermediates. The activation of the cAMP-responsive element binding protein (CREB) proceeds through phosphorylation by protein kinase A (16), which is activated by cAMP in response to activation of \( G_\alpha \). Ternary complex factors are activated by extracellular signal-regulated kinases, whose activation through Ras and/or Raf can be initiated by \( G_\beta\gamma \) released from \( G_\alpha \) or by \( G_{\alpha_\beta} \) (17–19). Activation of c-Jun is accomplished by c-Jun N-terminal kinases, which respond to \( G_\beta\gamma \), \( G_{\alpha_\beta} \), and \( G_{\alpha_\beta} \) through Cdc42 and Rac and sometimes Ras (20, 21). The activity of serum response factor is keyed to the depletion of G actin in response to activation of Rho (22) and can be achieved by \( G_\beta\gamma \), \( G_{\alpha_\beta} \), and \( G_{\alpha_\beta} \) (23). Other transcription factors regulated by G protein signaling include signal transducers and activators of transcription (STATs) (24) and nuclear factor \( \kappa B \) (25, 26).

The \( G_\alpha \) protein \( G_\alpha \) exists in platelets, neurons, and other highly differentiated cells exhibiting regulated exocytosis (27–30). The \( \alpha \) subunit of \( G_\alpha \) hydrolyzes GTP quite slowly and is the one member of the \( G_\alpha \) family that does not serve as a substrate for pertussis toxin. \( G_\alpha \) communicates with receptors that normally couple to \( G_\alpha \) (31, 32) and has been demonstrated to inhibit adenylyl cyclases I and V (33), to inhibit N-type Ca\(^{2+}\) channels (34), and to activate inwardly rectifying K\(^{+}\) channels (34, 35). Interactions of \( G_{\alpha_\beta} \) with a Rap1 GTPase-activating protein, the RGS protein RGSZ1, and inducers of neurite outgrowth have been demonstrated (12, 14, 36).

In an effort to identify effectors for \( G_\alpha \) that could play a role in hematopoietic development, we carried out a yeast two-hybrid screen for proteins in bone marrow cells that interact with activated \( G_{\alpha_\beta} \). We report one such protein here, Eya2. Eya2 is a human homologue of the Drosophila eyes absent gene product and interacts not only with \( G_{\alpha_\beta} \) but at least one other

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member of the Gα family, Gqα. Eya2 in combination with Six proteins is a transcription co-activator. Gαz and Gqα interact with Eya2 in mammalian cells, and this interaction represses the transactivating activity of Eya2 in part by inhibiting Six4-induced translocation of Eya2 from cytosol to the nucleus.

MATERIALS AND METHODS
cDNAs and Recombinant Plasmids—cDNAs used in this study were human Gα (57), GqαQ205L, Eya2 (38), and Six1 (39), mouse Six4 (40), and rat Gα (41), GqαQ205L, and GqαQ227L. Gα and the rat G protein subunit cDNAs were obtained from Drs. R. Fong (University of Southern California) and N. Dhanasekaran (Temple University), respectively. Eya2 and Six1 cDNAs were isolated from a human bone marrow cDNA library (CLONTECH, Palo Alto, CA) by the polymerase chain reaction based on published sequences. Flag (DYKDDDDK)-tagged Eya2 was generated by insertion of the Eya2 cDNA into pFlag-CMV2 (Eastman Kodak Co.). HA (YPYDVPDYA)-tagged proteins were generated by polymerase chain reaction using forward primers encoding the HA sequence and subsequent insertion of products into pcDNA3 (Invitrogen, Carlsbad, CA). The tags in both instances were placed at the N terminus. The glutathione S-transferase (GST)-Eya2 (269–538) fusion protein was generated by subcloning the Eya2 (269–538) cDNA into pGEX-5x-3 (Amer sham Pharmacia Biotech) immediately downstream of the GST sequence. The luciferase reporter construct for measuring Eya2/Six-mediated transcription was generated by cloning the BamHI-EcoRI fragment containing the adenovirus E1b TATA box from the pNFnLuc plasmid (Stratagene, La Jolla, CA) into pGLO (Promega, Madison, WI) to generate pGLO3-TATA; six copies of the MEF3 element (40) obtained by polymerase chain reaction were subcloned into pGLO3-TATA upstream of the TATA box (Sunluc/SnC1I sites). The sequences of all constructs were verified directly. pAS2–1 and pM (CLONTECH) are vectors used to express Gal4 DNA binding domain fusion proteins in yeast and mammalian cells, respectively. pVP16 (CLONTECH) is a vector used to express VP16-activating domain fusion proteins in mammalian cells.

**Yeast Two-hybrid Screen**—pG2–1-GaQ205L was used as a bait to screen a human bone marrow library (CLONTECH) by sequential transformation according to the manufacturer's protocol. Briefly, the yeast strain Y190 was transformed with pAS2–1-GaQ205L and plated on synthetic dropout agar plates lacking tryptophan. Control experiments confirmed that this construct had no autoactivating properties. Yeast surviving the selection protocol were then transformed with pACT2 bone marrow library plasmid DNA and plated onto synthetic dropout agar plates containing 2 mM 3-amino-1,2,4-triazole (Sigma) but lacking tryptophan, leucine, and histidin. Plates were incubated at 30 °C for 7 days, and subsequent colonies were transferred to paper filters for measurement of β-galactosidase activity according to the manufacturer's protocol. Library plasmids were isolated from positive colonies and used to re-transform Y190 together with pAS2–1-GaQ205L or, as a control, pAS2–1-p53 or empty plasmid to confirm the strength of interaction and facilitate isolation of single library plasmids. In other experiments the N- and C-terminal regions of Eya2 were subcloned into pACT2 for analysis of interactions with GqαQ205L, expressed by pAS2–1.

**Mammalian Two-hybrid Assay**—HeLa cells maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum were seeded in six-well plates (1 × 10⁶ cells/well) the day before transfection with 0.5 μg of the reporter gene construct pG3MEF3-Luc and the indicated expression plasmids (0.4 μg each); 0.01 μg of pRL-SV40 plasmid (Promega) was included as the internal control. 48 h after transfection, cell lysates were prepared, and luciferase activities were measured according to the manufacturer's protocol (Promega).

**Immunofluorescence Microscopy**—C2C12 cells were seeded into 12-well plates (3 × 10⁵ cells/well) and transfected with 0.5 μg of each of the indicated plasmids using Transfectam (Promega). The cells were replated 24 h thereafter onto collagen-coated glass coverslips for an additional 24 h, then fixed with 4% paraformaldehyde at room temperature for 10 min and permeabilized with 0.1% Triton X-100. Blocking was performed with 5% donkey serum and 10% fetal bovine serum in phosphate-buffered saline. The cells were then incubated for 2 h with one or more of the following antibodies in blocking buffer: mouse anti-Flag (M5, Sigma) at 25 μg/ml for detection of FLAG-tagged Eya2, chicken anti-HA (Aves Labs, Tigard, OR) at 1:100 for detection of HA-tagged Six4, rabbit anti-GaQ (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100, and rabbit anti-GaQ (1191Q205L), as bait and a human bone marrow cDNA library (3.5 million independent clones) as a source of interacting proteins; the Q205L mutation impairs the GTPase activity of GaQz (43). Sixteen positive clones were isolated in our screen from approximately 20 million transformants. Sequencing revealed that five of the clones were the GaQz-interacting protein GAP4 (44). The other 11 clones corresponded to Eya2, a human homologue of the Drosophila eyes absent (Eya) gene. Eya is a transcription co-activator that, as a complex with the sine oculis gene product So, is essential for eye specification and the development of other tissues in Drosophila (45, 46). Eya2 is one of four vertebrate forms of Eya (47, 48).

**RESULTS**

**GaQz and GqαQ205L Interact with Eya2**—In an attempt to identify novel effectors for GaQz, we performed a yeast two-hybrid screen using a constitutively active variant of GaQz, GqαQ205L (GaQzQL), as bait and a human bone marrow cDNA library (3.5 million independent clones) as a source of interacting proteins; the Q205L mutation impairs the GTPase activity of GaQz (43). Sixteen positive clones were isolated in our screen from approximately 20 million transformants. Sequencing revealed that five of the clones were the GaQz-interacting protein GAP4 (44). The other 11 clones corresponded to Eya2, a human homologue of the Drosophila eyes absent (Eya) gene. Eya is a transcription co-activator that, as a complex with the sine oculis gene product So, is essential for eye specification and the development of other tissues in Drosophila (45, 46). Eya2 is one of four vertebrate forms of Eya (47, 48).

The Eya2 clones contained two different lengths of inserts, one corresponding to amino acid residues 15–538 and the other to residues 141–538. Full-length Eya2 is 538 residues long and comprises N-terminal transactivation (1–269) and C-terminal "Eya" consensus (270–538) domains (Fig. 1). To locate the GαQz binding site on Eya2, the N- and C-terminal portions of Eya2 (residues 15–300 and 269–538, respectively) were fused to the GaQz activation domain and re-tested in yeast with GaQzQL again fused to the GaQz DNA binding domain. The C-terminal half of Eya2 alone was found to interact with GaQzQL.

To confirm the interaction between GaQzQL and full-length
Eya2 in mammalian cells and to test the specificity of the interaction, we employed a mammalian two-hybrid system. The constitutively active forms of Ga\(_a\) (Ga\(_a2\), Ga\(_a2\)Q205L), Ga\(_a1\) (Ga\(_a1\), Ga\(_a1\)Q227L), and Ga\(_a2\) (Ga\(_a2\), Ga\(_a2\)Q205L), as well as wild type Ga\(_a2\) and Ga\(_a2\)Q205L, were cloned into the Gal4 DNA binding domain vector pM, and the full-length Eya2 was cloned into the VP16 transcription-activating domain vector pVP16. The two expression plasmids were co-transfected into HeLa cells together with a pG5CAT reporter construct. We found that Ga\(_a2\), Ga\(_a1\), and Ga\(_a2\)Q205L interacted strongly with Eya2, as determined by CAT expression, whereas Ga\(_a2\)Q227L did not (Fig. 2, panel A). Wild type Ga\(_a2\) and Ga\(_a2\)Q205L subunits, which would assume an inactive conformation predominantly, interacted with Eya2 only very weakly. These data suggest that Eya2 is specific for the activated forms of these Ga\(_a\) family members.

We generated a GST-Eya2 C-terminal domain fusion protein to ascertain in pull-down experiments whether the interaction of Eya2 with Ga\(_a2\)Q205L and Ga\(_a2\)Q205L was direct (Fig. 2, panel B). In vitro translated [\(^{35}\)S]methionine-labeled, in vitro translated Ga\(_a2\)Q205L subunits (10% of starting material, left autoradiogram) were incubated with GST alone or GST-Eya2(269–538) attached to glutathione-agarose beads. The beads were pelleted, washed, and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The data represent one of three experiments providing equivalent results.

![Fig. 2. Interaction of Ga\(_a\) subunits with Eya2 in mammalian two-hybrid and GST pull-down assays. Panel A, Gal4 DNA binding domain-Ga\(_a\) subunit fusion protein plasmids were introduced together with the pG5CAT reporter gene by transfection into HeLa cells with or without the VP16 activation domain-Eya2 fusion protein plasmid. Forty-eight hours after transfection, cell lysates were prepared for analysis of CAT activity, which is expressed as the mean fold stimulation ± S.E. for three experiments. Panel B, \[^{[35}\)S\]methionine-labeled, in vitro translated Ga\(_a2\)Q205L subunits (10% of starting material, left autoradiogram) were incubated with GST alone or GST-Eya2(269–538) attached to glutathione-agarose beads. The beads were pelleted, washed, and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The data represent one of three experiments providing equivalent results.](image-url)

**Interaction of Ga\(_a\) and Ga\(_a\) with Eya2**

Eya2 interacts with Six Proteins—Eya2 also interacts with “Six” proteins, which are mammalian homologues of So and are reported to promote translocation of Eya2 to the nucleus (49). The Six proteins consist of a Six consensus domain near the N terminus, an adjacent homeodomain, and a variable C terminus, which in some instances (e.g., Six4) contains a transactivation domain (Fig. 3, panel A). We carried out a mammalian two-hybrid assay to confirm the interaction between Eya2 and Six proteins using Six1 as the representative (panel B). Six1 had not been evaluated previously. Six1 and the isolated consensus Six domain, both presented as Gal4 DNA binding domain fusion proteins, interacted with Eya2. We also evaluated interactions using a reporter gene (luciferase) under control of a MEF3/TATA promoter. Six proteins alone, to some extent, but the combination of Six proteins with Eya2, to a much greater relative extent, promoted gene expression (panel C); the combination of Six4 and Eya2 was particularly effective in this regard.

**The Binding of Ga\(_a\) Subunits and Six Proteins to Eya2 Is Mutually Exclusive**—We asked whether Six1 and Six4 have any effect on the interaction of Eya2 with Ga\(_a\)QL subunits, and vice versa. The mammalian two-hybrid assay was performed using Ga\(_a\)QL and Ga\(_a\)QL subunits fused to the Gal4 DNA binding domain and Eya2 fused to the VP16 activation domain; Six1 and Six4 under the control of a cytomegalovirus promoter were also introduced (Fig. 4, panel A). As shown, both Six1 and Six4 suppressed the interaction of the Ga\(_a\)QL subunits with...
Interaction of $G_{\alpha_z}$ and $G_{\alpha_i}$ with Eya2

Eya2. Six1 and Six4 had no effect on the interaction of p53 with the SV40 large T antigen presented as DNA binding and activation domain fusion proteins (not shown), supporting specific-
also demonstrated that Eya2 and Six proteins act cooperatively in transactivation.

Our interest in Eya2 was prompted by identification of Eya2 cDNA sequences in a yeast two-hybrid screen with Ga\textsubscript{i2}QL as bait. Eya2 was one of two proteins identified in the screen; the other was GAIP (44). Three other proteins have been reported previously to interact directly with activated Ga\textsubscript{z}: RGSZ1, Rap1GAP, and GRIN (12, 14, 36). RGSZ1 and Rap1GAP were cloned in yeast two-hybrid screens using Ga\textsubscript{z}QL and a human brain cDNA library, whereas GRIN was cloned from a mouse embryo cDNA expression library using G\textsubscript{a}GTP\gamma S as a probe. Although the number of transformants screened in our assay was 5-fold the number of independent clones, some interacting proteins may have escaped detection due to the heterogeneity in cell populations contributing to the library. We find no apparent difference in the strength of interactions of Eya2 with Ga\textsubscript{a}QL and Ga\textsubscript{z}QL, which is an observation similar to that published for GAIP. RGSZ1, on the other hand, prefers Ga\textsubscript{a}QL to Ga\textsubscript{z}QL (36), whereas Rap1GAP prefers Ga\textsubscript{a}QL to all other subunits (12).

Yeast two-hybrid screens for interacting proteins have been applied to a variety of G protein subunits. Active subunit conformations are not always required. Rap1GAP is reported to interact preferentially with unactivated Ga\textsubscript{z} (19), whereas calnuc (nucleobindin) interacts with Ga\textsubscript{z} (55), and Purkinje cell protein-2 interacts with Ga\textsubscript{z} (56) regardless of subunit activation state. Yeast two-hybrid techniques have also been used to demonstrate interactions between Ga\textsubscript{z} subunits and Raf (6), the ankyrin repeat-containing protein (57), RhoA and Cdc42 (9), and N-type calcium channel sequences (58).

That the interaction of Ga\textsubscript{a} or Ga\textsubscript{z} with Eya2 requires an active conformation is consistent with the idea that Eya2 is an effector, but not proof. In first assessing the probability of a normal interaction between the two proteins, our concern was that Eya2 would exist primarily in the nucleus, as does Drosophila Eya. However, our work and that published by Ohoto et al. (49) show that Eya2 expressed alone assumes a cytoplasmic distribution. Its co-fractionation with cytosolic protein following cell lysis suggests that it is in fact diffusible. These findings are perhaps not unexpected. While Drosophila Eya has a consensus nuclear localization signal, the vertebrate proteins do not, suggesting differences in functions related to subcellular location (38). Eya2 would have ample opportunity as a cytosolic protein, as do several G protein effectors, to interact with Ga subunits.

Six proteins provide the means by which Eya2 can be transported into the nucleus and affixed to DNA in order to assume a transactivating role. A reasonable question is whether Ga\textsubscript{z}QL and Ga\textsubscript{a}QL can effectively compete with Six proteins in this process. The data presented here demonstrate that they can. In the MEF3/TATA transactivation assays in HeLa cells and in the immunofluorescence experiments with C2 myoblasts, the Ga\textsubscript{z}QL subunits almost completely negated the actions of Six1 and Six4. The antagonism is not one-sided, however, as Six1 and Six4 can disrupt the interaction between Eya2 and Ga\textsubscript{z}QL subunits in the mammalian two-hybrid assay. Although much remains to be understood about the presentation of Eya2 to appropriate gene regulatory elements, we suspect that Ga\textsubscript{z} and Ga\textsubscript{a} can regulate gene transcription through Eya2 in several ways. The activation of Ga\textsubscript{a} or Ga\textsubscript{z} in the intact cell by agonist-activated receptors involves conformational changes in Ga\textsubscript{a} and Ga\textsubscript{z} coincident with GTP binding. Subunits assuming an active conformation could bind cytosolic Eya2 at membranes or, under circumstances described for at least one other subunit (Ga\textsubscript{i2}) (59), conceivably in the cytosol. If Eya2 is normally carried to the nucleus by Six proteins in a constitutively or regulated fashion, activated Ga\textsubscript{a} or Ga\textsubscript{z} would be anticipated to interrupt that process and thereby diminish the amount of Eya2 entering the nucleus. Additionally, Eya2 and Six proteins in the nucleus would probably not remain locked together as a transactivating complex. Ga\textsubscript{a} or Ga\textsubscript{z} might therefore decrease nuclear Eya2 based on the equilibrium established between nuclear and cytosolic forms of the protein. In either case, any gene activity under the control of Eya2 would be diminished. Ga\textsubscript{a} hydrolyzes GTP much more slowly than most other Ga subunits (60), a property conducive to sequestration of Eya2. G protein subunits of the G\textsubscript{i} family, moreover, are reported to translocate or diffuse into the nucleus in certain instances.
Interaction of Ga\textsubscript{a} and Go\textsubscript{a} with Eya2

(61–63), which may also be relevant to interactions of the subunits with Eya2. In our studies, we did not distinguish between monomeric cG\textsubscript{DP} and cG\textsubscript{D}P complexed with G\textsubscript{z} as the inactive forms of wild type subunits; we suspect that both forms of subunit cannot interact with Eya2 and that interaction is the property of the active form only.

Six proteins need not always be considered. For cells lacking Six proteins, Eya2 would be completely cytosolic, and the interaction of activated Ga subunits with Eya2 could be envisioned in much the same manner as that for the several already documented effectors and RGS proteins. In this context, Eya2 could exhibit activities apart from those of transactivation. Conversely, and just as significantly, Eya2 may prove relevant to the activation state of Ga\textsubscript{a} and Go\textsubscript{a} or the ability of these subunits to interact with other effectors.

We believe that the inhibition of Eya2-Six1/4-activated MEF3/TATA reporter gene activity by Go\textsubscript{a}QL and Go\textsubscript{a}QL is most likely due to the inhibition of Eya2 translocation into the nucleus. Other routes of inhibition, for example through Go subunit-regulated second messenger pathways, remain possible. Go\textsubscript{a}QL, which by two-hybrid analysis does not interact with Eya2 and by immunofluorescence does not disrupt the nucleus. Other routes of inhibition, for example through Ga\textsubscript{a}z complexed with G\textsubscript{i2} or the ability of these subunits with Eya2. In our studies, we did not distinguish (61–63), which may also be relevant to interactions of the several already documented effectors and RGS proteins. In this context, Eya2 could exhibit activities apart from those of transactivation.

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