Inhibition of Human HL-60 Cell Responses to Chemotactic Factors by Antisense Messenger RNA Depletion of G Proteins*

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Chemoattractant factors bound to receptors of the seven-transmembrane domain family signal leukocytes through associated guanine nucleotide-binding (G) proteins. Human leukocytes of the HL-60 line, which express G protein-coupled receptors for leukotriene B$_4$ (LTB$_4$) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) after differentiation with vitamin D$_3$ and transforming growth factor-β, were transfected with expression plasmids containing antisense-oriented cDNAs encoding the α-chains of Go, Gi$_1$, Gi$_2$, and Gi$_3$. Antisense mRNA for G$_α$ and G$_α$ α-chains suppressed by over 80% the level of the respective G protein. G$_α$-deficient HL-60 cells had depressed functional and intracellular calcium responses to LTB$_4$ and fMLP, but no alterations in the responses of cyclic adenosine 3',5'-monophosphate (cAMP). In contrast, HL-60 cells deficient in Gi$_3$ lost only responses of the intracellular concentration of cAMP. Antisense mRNA suppression of distinct G proteins thus may delineate some transductional requirements for cellular responses.

Heterotrimeric guanine nucleotide-binding proteins (G proteins)1 transduce signals from ligand-occupied cellular membrane receptors to a wide-range of functional effectors (1–3). The large family of cellular receptors, that utilize one or more chains of G proteins for signal transduction, share both the general topological property of seven membrane-spanning domains and substantial homology of amino acid sequence (4). Current evidence suggests that more than one type of G protein associate with some receptors and may transmit distinct signals to different effectors, as well as additive or opposing signals to the same effector. The physical coupling of two or more different G proteins to one receptor was shown by immunoprecipitation of solubilized receptors with antibodies specific for G proteins (5, 6) and by anti-G protein antibody-mediated uncoupling of receptor-G protein complexes (7, 8). The functional interactions of one receptor with multiple G proteins have been demonstrated by introduction into intact cells of anti-G protein antibodies by patch-clamp pipettes (9) and of antisense oligonucleotides (10, 11), application of anti-G protein antibodies in cell membrane preparations (12, 13), and specific competition by a novel peptide antagonist for G protein binding to recombinant receptors in phospholipid vesicles (14). Interpretation of the results of many past studies, however, have been complicated by the limitations of specificity and intracellular access of some immunochromep probes, only partial effects of antisense oligonucleotides and lack of concurrent assessment of functional and biochemical effects of alterations in G proteins. Transfection of human HL-60 cells with expression plasmids encoding full-length antisense (AS) messages, sufficient to suppress each G protein α-chain selectively, now is shown to identify their receptor-specific signals.

EXPERIMENTAL PROCEDURES

Construction of Antisense cDNA Plasmids—Four full-length cDNAs encoding different G proteins were provided by Dr. R. R. Reed (Johns Hopkins University School of Medicine) (15). EroII-EcoRI fragments containing the full-length coding region and additional 5'- and 3'-untranslated sequences for the α-chains of G$_α$, G$_i_2$, G$_s$, and G$_z_3$ were separately inserted into the EcoRI site of pcDNAI (Invitrogen) in reverse orientation to the direction of transcription of the cytomegalovirus promoter. The structure of each construct was verified by direct sequence analysis.

Culture, Transfection, and Differentiation of HL-60 Cells—HL-60 cells were cultured in complete RPMI medium, as described (16), washed and suspended at 1 x 10$^6$/ml in 5–10 ml of serum-free Opti-MEM (Life Technologies, Inc.), and transfected with antisense cDNA by incubation with 150–200 µg of plasmid DNA and 120 µl of DOTAP (Boehringer Mannheim) for 6 h at 37 °C in 5% CO$_2$:95% air. An equal volume of complete medium was added and the incubation continued for 18 h. The HL-60 cell transfectants then were induced to differentiate by washing and resuspension at 1 x 10$^6$/ml in complete medium containing 100 nM 1,25-dihydroxyvitamin D$_3$ and 1 ng/ml human purified recombinant transforming growth factor-β (TGF-β; Austral Biologicals), and further culture for 3–14 days. The density of HL-60 cells in culture increased at 4 days to means of 2.3, 2.5, and 2.7 x 10$^6$/ml, respectively, for sham (vector only), G$_α$-antisense (AS), and G$_z_3$-AS transfectants, and at 8 days to 4.0, 4.0, and 4.3 x 10$^6$/ml (n = 3). Thus the extent of differentiation, assessed by α-naphthyl esterase staining (16), was 16, 17, and 24% at 4 days for sham, G$_α$-AS, and G$_z_3$-AS transfectants, respectively, and 67, 64, and 71% at 8 days (n = 3). Thus neither AS construct altered cell growth or differentiation significantly.

Analysis of G Proteins by Western Immunoblots—Replicate suspensions of 3 x 10$^6$ differentiated transfectants in 1 ml of Ca$^{2+}$- and Mg$^{2+}$-free Hanks' solution (HBSS$^-$) were incubated with 0.3 µM diazisopropyl fluorophosphate (Sigma) for 2 h at room temperature, washed twice in 2 ml of HBSS$^-$, resuspended in 0.3 ml of 0.1 x sucrose containing 1 µM phenylmethylsulfonyl fluoride, 2 µM EDTA, and 5 mM Tris-HCl (pH 7.2), and disrupted in a chilled glass tube homogenizer. Fifteen-pl aliquots of the 1,000 x g supernatant of each homogenate were diluted 1:5 and 1:20 and mixed with an equal volume of Laemmli's solution (17), and then subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 20 min and 160 V for 90 min.
The proteins resolved were transferred to nitrocellulose and developed with a 1:1,000 dilution of rabbit antibodies to the carboxyl-terminal peptides 345-354 of the G\textsubscript{1}\textgamma\textsubscript{1} and G\textsubscript{1}\textgamma\textsubscript{2} \textalpha-subunits (Calbiochem, Inc., La Jolla, CA), or with a 1:500 dilution of rabbit antibody to bovine brain G\textsubscript{1}\textgamma\textsubscript{1} \textalpha-subunit (Dr. Su-Chen Tsai, National Institutes of Health). Bound antibodies were detected with a luminescence detection system (Immun-Lite, Bio-Rad).

Assessment of HL-60 Cell Binding and Effects of Leukotriene B\textsubscript{4} (LTB\textsubscript{4}) and N\textsubscript{6}Formyl-methionyl-leucyl-phenylalanine (fMLP)—The binding of \textsuperscript{3}H-LTB\textsubscript{4} (Amersham Corp.) and \textsuperscript{3}HfMLP (DuPont NEN) to differentiated control and transfected HL-60 cells was quantified as described (16), using GPCR glass fiber filters (Whatman) to resolve bound from unbound radioactivity. Chemotactic responses of 2 \texttimes 10\textsuperscript{6} differentiated transfected cells/100 ml HBSS with 0.05 g/100 ml ovalbumin and 25 mM HEPES (pH = 7.3) in each chamber (Adaps, Dedham, MA) were quantified after 2 h at 37°C. A 8-μm diameter pore filters were washed and stained, and the number of HL-60 cells per high power field above the background in filters from chambers without a stimulus were enumerated microscopically, as described (16, 18, 19). To examine lysosomal granulation, suspensions of 4 \times 10\textsuperscript{6} differentiated transfected cells/100 ml HBSS with 0.1 g/100 ml ovalbumin, 5 μg/ml cytochalasin B (Sigma), and 10 mM HEPES (pH = 7.4) for 25 min at 37°C in HBSS containing 0.1 g/100 ml ovalbumin and 25 mM HEPES (pH = 7.3). The Fura 2-loaded transfected cells were washed, resuspended at 10\textsuperscript{7}/ml in phosphate-buffered saline, and warmed to 37°C. Fluorescence was quantified in 1-ml portions of suspensions from 1 min before to 5 min after the addition of 1 μM LTB\textsubscript{4} or 1 μM fMLP in a stirred and 37°C controlled cell of a Perkin-Elmer model 650-40 fluorimeter, as described (16). Maximum and minimum fluorescence were determined by the respective additions of 200 μl of Triton X-100 in distilled water (1 g/100 ml) and then 20 μl of 0.5 M EGTA, at 5 min after addition of the stimulus. To quantify intracellular concentration of cAMP, 0.5-ml duplicate suspensions of 2 \times 10\textsuperscript{6} HL-60 cells in HBSS with 0.1 g of ovalbumin/100 ml, 10 mM HEPES (pH = 7.3), and 100 μM isobutyl methylxanthine and Ro 1724 (Calbiochem) were preincubated for 10 min at 37°C, stimulated as indicated, and were incubated for 2 min at 37°C. The responses were stopped by addition of cold ethanol 10%, and cyclic AMP in the 3,000 x g supernatants was quantified by radioimmunoassay after acetylation of cyclic AMP according to the manufacturer's protocol (DuPont NEN).

RESULTS AND DISCUSSION

Lipotransfection of human HL-60 cells with the high level expression vector pcDNA I, carrying cDNA that encodes full-length AS messages for the \textalpha-chains of G\textsubscript{1}\textgamma\textsubscript{1}, G\textsubscript{1}\textgamma\textsubscript{2}, G\textsubscript{1}\textgamma\textsubscript{3}, and G\textsubscript{0}, and alone, in combination, suppressed the cellular level of the respective G proteins. The extent of suppression of G protein \textalpha-chains was quantified by SDS-PAGE and Western blot analysis of extracts of an equal number of each transfectant and of control HL-60 cells sham-transfected with vector alone (Fig. 1). The limit of detection of G protein \textalpha-chains by Western blot was approximately 5% of the total present in undiluted extracts of sham-transfected HL-60 cells. The two anti-carboxyl-terminal peptide G protein antibodies used detect common antigens in G\textsubscript{1}\textgamma\textsubscript{2} and G\textsubscript{1}\textgamma\textsubscript{1} and in G\textsubscript{1}\textgamma\textsubscript{3}, respectively. Maximal suppression of the cellular content of G\textsubscript{1}\textgamma\textsubscript{2} + G\textsubscript{1}\textgamma\textsubscript{1} by G\textsubscript{1}\textgamma\textsubscript{2} AS and G\textsubscript{1}\textgamma\textsubscript{3} by G\textsubscript{1}\textgamma\textsubscript{3} AS, respectively, was 80-95% and 80% at 6-7 days (Fig. 1) Western blot analyses of the course of suppression of G\textsubscript{1}\textgamma\textsubscript{2} + G\textsubscript{1}\textgamma\textsubscript{1} and G\textsubscript{1}\textgamma\textsubscript{2} + G\textsubscript{1}\textgamma\textsubscript{3} protein \textalpha-chains showed less than half of the maximum at 3-4 days, no greater suppression at 10 days, and loss of more than half of the maximum effect after 14 days (n = 2). The time for attainment of the greatest suppression presumably reflects a requirement for catalysis of existing G proteins, after inhibition of synthesis by antisense mRNA.

G\textsubscript{1}\textgamma\textsubscript{1} is not reflected in analyses of G\textsubscript{1}\textgamma\textsubscript{2} (Fig. 1, panel A), as expected from the lack of detection of G\textsubscript{1}\textgamma\textsubscript{1} protein (20) and of G\textsubscript{1}\textgamma\textsubscript{1} mRNA in previous Northern blot analyses of HL-60 cells. In contrast, residual \textalpha-chain immunoreactivity apparently attributable to unsuppressed G\textsubscript{0}, as determined by Western blotting of extracts from sham-transfected HL-60 cells. The rabbit antibodies recognized G\textsubscript{1}\textgamma\textsubscript{3} and G\textsubscript{1}\textgamma\textsubscript{2} \textalpha-subunits (A) or the G\textsubscript{1}\textgamma\textsubscript{2} and G\textsubscript{1}\textgamma\textsubscript{3} \textalpha-subunits (B). Lanes 2, 1, and 4 of each panel contained undiluted extract of sham transfectants and dilutions of 1:20 and 1:5, respectively, and lane 3 contained undiluted extracts of G\textsubscript{1}\textgamma\textsubscript{2}-deficient (A) or G\textsubscript{1}\textgamma\textsubscript{3}-deficient (B) transfectants.
reflect no statistically significant differences. Similarly, AS transfections did not alter significantly the total number of receptors or the number of high affinity receptors for LTB4 and fMLP. The respective high affinity site responses of Go-deficient transfectants were attributable to ab-

Go-AS, and Gi2-AS transfectants were on Go protein at high concentrations of LTB, could be attrib-

that of sham transfectants. Pertussis toxin alone had never the highest concentration of LTB4 was indistinguishable from AS mRNA and the apparently lesser dependence of chemotaxis in Go-deficient transfectants was suggested by depressed func-

anomalies in binding of the chemoattractants was excluded by the results of three studies of binding of [3H]LTB4 and [3H]fMLP. The respective high affinity site Kc values of sham, Gc-AS, and Gc-AS transfectants were 10, 7.6, and 8.2 nM for LTB4, and 4.7, 5.9, and 4.8 nM for fMLP (mean, n = 3), which reflect no statistically significant differences. Similarly, AS transfections did not alter significantly the total number of receptors or the number of high affinity receptors for LTB4 and fMLP detected in the same studies of binding.

A disturbance of one or more signal transduction pathways in Gc-deficient transfectants was suggested by depressed func-

Fig. 3. Effects of antisense mRNA depletion of individual G protein α-chains on β-glucuronidase release by HL-60 cells. Each bar and bracket depicts the mean and S.D. of the results of three studies performed in duplicate and expressed as a percentage of the release of β-glucuronidase by identically stimulated sham transfectants. G0-AS, G1-AS, G2-AS transfectants. The mean percentage release values (± S.D.) of β-glucuronidase by sham transfectants stimulated with 0.1 and 1 μM LTB4, 0.1 and 1 μM fMLP, and 1 μM A23187, respectively, were 9.9 ± 2.1, 10.4 ± 2.6, 8.8 ± 3.8, 13 ± 3.3 and 10.7 ± 2.6, above a background of release of 5.8-10.2% in buffer alone. A two-sample f test was used to assess the significance of the decreases in β-glucuronidase release, compared to that by sham transfectants. *, p < 0.01; +, p < 0.05; AS, antisense.

Fig. 4. Decrease in the response of [Ca2+]i in HL-60 cells to chemotactic stimuli by mRNA antisense depletion of Gc-protein α-chains. The tracings shown are representative of two to four others for each subset of samples. The concentration of intracellular Ca2+ was calculated as described (16, 23).

Table I
Prevention of chemotactic factor-induced increases in [Ca2+]i in differentiated HL-60 cells by antisense mRNA suppression of Gc-protein α-chains

| G protein α-chains | Chemotactic stimulus | [Ca2+]i (%) | % of sham transfectant |
|-------------------|----------------------|-------------|----------------------|
| G11 + G12 + G13 + Gc | LTB4 | 35 ± 30 (4)* | 35 ± 20 (4) |
|                   | fMLP | 49 ± 21 (4)  | 49 ± 21 (4)  |
| Gc                 | LTB4 | 30 ± 16 (2)  | 30 ± 16 (2)  |
|                   | fMLP | 48 ± 28 (2)  | 48 ± 28 (2)  |
| G11 + G12 + G13    | LTB4 | 95 ± 20 (2)  | 95 ± 20 (2)  |
|                   | fMLP | 85 ± 21 (2)  | 85 ± 21 (2)  |

* Each value is the mean ± S.D. of the results of the number of studies shown in parentheses; the values in the upper four columns are significantly suppressed, with p < 0.05 by a paired t test.

The partial inhibitory effectiveness of Gc-specific antisense mRNA depletion of individual G proteins (25). The lysosomal degranulation of HL-60 cells through G protein α-chains.

Each value is the mean ± S.D. of the results of number of studies shown in parentheses; the values in the upper four columns are significantly suppressed, with p < 0.05 by a paired t test.

Table I
Prevention of chemotactic factor-induced increases in [Ca2+]i, in differentiated HL-60 cells by antisense mRNA depletion of Gc-protein α-chains
Antisense Messenger RNA Depletion of G Proteins

TABLE II
Prevention of LTB4-induced decreases in cAMP concentrations in differentiated HL-60 cells by AS mRNA suppression of Gα protein α-chains

| G protein α-chains depleted by transfection with antisense message | Concentration of cyclic AMP (mean ± S.D.) pmol/10^6 HL-60 cells |
|---------------------------------------------------------------|---------------------------------------------------------------|
|                                 | Buffer alone | LTB4 (10 nM) | Forskolin (10 μM) | Forskolin + LTB4 |
| Sham (vector only)           | 1.9 ± 0.2    | 2.6 ± 0.3    | 12.9 ± 2.3         | 5.1 ± 1.2*       |
| Gα                           | 2.0 ± 0.1    | 4.1 ± 1.6    | 23.6 ± 5.5         | 5.6 ± 0.6**      |
| G2a                          | 2.1 ± 0.5    | 5.9 ± 1.6    | 38.7 ± 11.6        | 25.6 ± 5.6 (NS)  |

Increased substantially by the co-transfection of antisense messages directed to G11-3 (Fig. 4). In additional studies, only Gα deficiency suppressed significantly the [Ca^{2+}], responses of HL-60 cell transfectants to LTB4 and fMLP (Table I). The biochemical consequences of G2α deficiency in HL-60 cell transfectants was examined in relation to the known inhibition of adenylyl cyclase activity mediated by G1 proteins (1-3). LTB4 inhibited by 75% or more the forskolin-induced increase in concentration of cAMP in sham and G2α-deficient transfectants, but only inhibited by a mean of 40% the increase observed in G2α-deficient HL-60 cells (Table II).

The application of antisense mRNA approaches to suppress selectively the levels of individual G proteins in HL-60 cells, bearing receptors for LTB4 and fMLP after differentiation, allows assignment of the specific roles of some G proteins in signal transduction. High affinity binding of chemotactic factors was less dependent on G proteins than signal transduction. Gα appears to mediate increases in [Ca^{2+}], that are coupled to both chemotactic and degranulation responses, whereas the inhibition of adenylyl cyclase attributable to G2α had no detectable involvement in either functional response.

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