Pertussis toxin inhibits chemotaxis of neutrophils by preventing chemoattractant receptors from activating trimeric G proteins in the G_i subfamily. In HEK293 cells expressing recombinant receptors, directional migration toward appropriate agonist ligands requires release of free G protein βγ subunits and can be triggered by agonists for receptors coupled to G_i but not by agonists for receptors coupled to two other G proteins, G_s and G_q. Because activation of any G protein presumably releases free Gβγ, we tested the hypothesis that chemotaxis also requires activated α subunits (Gα_i) of G_i proteins. HEK293 cells were stably cotransfected with the G_i-coupled receptor for interleukin-8, CXCR1, and with a chimeric Gα_iGα_qz5, which resembles Gα_i in susceptibility to activation by G_i-coupled receptors but cannot regulate the Gα_i effector, adenylyl cyclase. These cells, unlike cells expressing CXCR1 alone, migrated toward interleukin-8 even after treatment with pertussis toxin, which prevents activation of endogenous Gα_i but not of Gα_iGα_qz5. We infer that chemotaxis does not require activation of Gα_i. Because chemotaxis is mediated by Gβγ subunits released when G_i-coupled receptors activate Gα_qz5, but not when Gα_q or G_i-coupled receptors activate their respective G proteins, we propose that G_i-coupled receptors transmit a necessary chemotactic signal that is independent of Gα_i.

As it migrates to a site of infection or tissue injury, an inflammatory cell must detect a chemokine gradient and organize its cytoskeleton to move in the right direction (1–4). The signaling pathways responsible for this complex cellular response are poorly understood. Pertussis toxin, which specifically prevents receptor-dependent activation of G_i proteins, blocks chemotactic migration of neutrophils; we therefore infer that G_i proteins play essential roles in mediating the chemotactic signal. Activation of G proteins by serpentine receptors releases two potential stimulators of downstream signals, an α subunit (Gα_i) bound to GTP, and a free Gβγ subunit (5). For example, the α_i subunits of G_i proteins directly mediate inhibition of adenylyl cyclase, while the βγ subunits of these proteins mediate opening of K+ channels and stimulation of phospholipase Cβ (6).

To identify the G protein subunit that mediates chemotaxis, we have begun to study chemotaxis in a cell line, HEK293,1 which is amenable to stable transfection with normal and mutant receptors and other signaling proteins. Endogenous Gα_i subunits of HEK293 cells include α_i6, α_i9, α_i11, α_i9q, but not α_5 or α_i7 (7, 8). In this model we found that chemotaxis requires receptors that activate G_i and that release of free βγ is essential (9). Receptors that activate two other G proteins, G_s and G_q, could not mediate chemotaxis in HEK293 cells. Abundant evidence indicates that activation of these two G proteins, like that of Gα_i, involves dissociation of Gβγ from GTP-bound Gα_i. Accordingly, it is reasonable to ask why the Gβγ released from α_i-GTP or α_q-GTP could not mimic the chemotactic effect of Gβγ released from α_q-GTP.

One answer is that Gα_i itself makes the difference, by activating an essential downstream signal distinct from those triggered by Gβγ. A second possibility is that G_i proteins are simply more abundant than G_s or G_q, and accordingly release more Gβγ upon activation. A third possibility is that inhibitory signals generated by the α_i subunits of G_s or G_q block the chemotactic response to free βγ. To test these possibilities, which are not mutually exclusive, we assessed chemotaxis of HEK293 cells expressing different combinations of receptors and G proteins. Our results show that chemotaxis requires a receptor that can activate G_i but does not require Gα_i itself. We propose that chemotaxis requires not only Gβγ, but also a signaling function of G_i-coupled receptors that is distinct from activation of Gα_i.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant IL-8, forskolin, and rat collagen type I were obtained as described (9). The modified Boyden chamber was purchased from Poretics. Pertussis toxin was obtained from List Biologicals. PC3 stable transfectants were cotransfected with the various constructs in pcDNA1 and pcDNA3 was obtained from Israel Charo, Gladstone Institute, San Francisco General Hospital (11). The interleukin 8 receptor type A (hereafter termed CXCR1) subcloned into pcDNA1 was obtained from Israel Charo, Gladstone Institute, San Francisco General Hospital.

**Cell Culture and Transfection**—HEK293 cell lines stably expressing CXCR1 and the m3-muscarinic acetylcholine receptor (m3mAChR) were generated as described (9) and maintained in G418 (800 μg/ml). For double stable transfectants, a vector containing a hygromycin resistance cassette was cotransfected with the various Gα constructs in pcDNA1. Clones stably expressing both CXCR1 and the respective Gα constructs were selected and maintained in both G418 and hygromycin (200 μg/ml). Cell lines were propagated as described (9).

**Assays**—Assays of chemotaxis, cAMP accumulation, and inositol phosphate accumulation were performed as described (9).

**Immunoblots**—Subconfluent cells (5 × 10^6) were lysed in RIPA

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§ The abbreviations used are: HEK, human embryonal kidney; IL-8, interleukin 8; CXCR1, interleukin 8 receptor type A; m3mAChR, m3-muscarinic acetylcholine receptor; GRK, G protein receptor kinase.
buffer containing 5% Nonidet P-40, 2.5% deoxycholate, 250 mM Tris (7.4), 750 mM NaCl, and 12.5 mM MgCl2 for 30 min at 4 °C. Lysates were diluted as noted in RIPA buffer, separated on a 12% SDS gel, and transferred to polyvinylidene difluoride membranes. Membranes were probed with EE monoclonal antibody as described (10). ECL (NEN Life Science Products) was used to visualize immunoreactive bands.

RESULTS

Abundant evidence indicates that receptor activation of all trimeric G proteins causes dissociation of Gβγ from Goq-GTP. If so, why does receptor activation of Gi elicit chemotaxis, but release of Gβγ from activated Gi or Gq does not (9, 12)? One trivial explanation is that the second messengers synthesized in response to activation of Gi and Gq actually inhibit the chemotactic response that would otherwise be elicited by release of Gβγ. Fig. 1 shows that this explanation could account for the failure of activated Gi, but not that of activated Gq, to mediate chemotaxis. Forskolin, which reproduces the stimulatory response to IL-8 in HEK293 cells expressing the recombinant IL-8 receptor, CXCR1 (Fig. 1A); this result is in accord with previous observations (13–15) that cAMP inhibits the chemotactic response of neutrophils and other cells. To test whether activated Goq can inhibit chemotaxis, we cotransfected cells expressing recombinant CXCR1 with a cDNA (10) encoding functionally active Goq (Goq-Q205L). Expression of Goq-Q205L increased basal phosphoinositide accumulation more than 30-fold (result not shown), but had no effect whatever on chemotaxis toward IL-8 (Fig. 1B).

If activated Goq cannot inhibit chemotaxis, we must ask why the release of Gβγ from receptor-activated Gi does not mediate chemotaxis. The simplest explanation would be that chemotaxis requires Goq-GTP, as well as Gβγ. Accordingly, we asked whether CXCR1 can elicit chemotaxis when it activates a G protein containing a chimeric Ga, Goqα5 (11), which cannot regulate activity of a direct effector of Ga, adenyl cyclase. Goqα5 is identical to Goq except that its C-terminal five amino acids are replaced by the corresponding sequence of Ga, a Ga that responds to stimulation by Gi-coupled receptors but is not inhibited by treatment with pertussis toxin (16). Expression of recombinant Goq with CXCR1 conferred on the cells the ability to migrate toward IL-8 even after treatment with pertussis toxin (result not shown); this result did not speak to the question of whether Goqα5 is required for chemotaxis, however, because Goq can mimic the inhibitory effect of Goq on adenyl cyclase (16).

We have shown that ligand-bound Gi-coupled receptors can use Goqα5 to activate the phosphoinositide pathway usually regulated by Goq (11). Cells that co-expressed Goqα5 and CXCR1 migrated toward IL-8 (Fig. 2A). Chemotaxis was mediated by a G protein containing Goqα5, rather than by endogenous Gi, as shown by the inability of pertussis toxin to prevent chemotaxis of Goqα5-expressing cells; the toxin completely blocked chemotaxis toward IL-8 in control cells expressing

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**Fig. 1. Effect of concurrent activation of Goq and Goqz5 on chemotaxis toward IL-8.** Migration assays were performed in a 48-well Boyden chamber, as described (9), on cells stably expressing CXCR1 alone (filled and open squares; panel A) or CXCR1 plus vector (open squares; panel B) or plus Goqα5-Q205L (open circles; panel B). Forskolin (open squares, panel A) was present at 200 μM. Values represent the mean ± S.E. of six determinations. Similar results were obtained in three or more independent experiments.

**Fig. 2. Effect of overexpressed Goqα5 on chemotactic and second messenger responses to IL-8.** A and B. Migration assays were performed as described (9) on cells expressing either CXCR1 with vector (squares, panels A and B) or CXCR1 with Goqα5 (open triangles, panel A). Cells were incubated without (filled squares, panel B) or with (other symbols, panels A and B) 500 μg/ml pertussis toxin for 4 h at 37 °C. The cells were subsequently washed and assayed as described. C. Accumulation of inositol phosphates in response to the indicated concentrations of IL-8 was measured. CXCR1 alone, diamonds; CXCR1 plus Goqα5, squares. Cells were treated with (open symbols) or without (filled symbols) pertussis toxin, as described for panels A and B. D. IL-8 (10 ng/ml) inhibition of cAMP accumulation stimulated by forskolin (200 μM) in CXCR1-expressing cells. Cells expressed CXCR1, with or without Goqα5, and were treated with or without pertussis toxin, as indicated in the figure. Values represent percent inhibition by IL-8 of the forskolin-stimulated cAMP response. Forskolin alone increased cAMP more than 100-fold. Values represent the mean ± S.E. of six determinations for panels A and B and three determinations for panels C and D. Similar results were obtained in three or more independent experiments.
pressed in these cells. Thus, it is possible that activation of G_q
binant G_a clonal antibodies against epitopes inserted into both G_types of cell (Fig. 3)
coupled receptors may activate a specific subset of G_a only with the m3AChR cDNA, unlike the presumably larger
amount of G_a in transfected cells, cannot release sufficient amounts of G_βγ in response to receptor stimulation.

We considered a potential quantitative explanation for the previously reported (9) failure of a G_q coupled receptor, the
m3AChR, to mediate chemotaxis in HEK293 cells. Although the cellular content of G_q in HEK293 cells is unknown, it is
probably lower than that of the exogenous G_q stably expressed in these cells. Thus, it is possible that activation of G_q
does release G_βγ, but that the amount of membrane-bound G_q:G_βγ available for receptor activation in cells transfected
only with the m3AChR cDNA, unlike the presumably larger amount of G_q:G_βγ in transfected cells, cannot release sufficient
amounts of G_βγ in response to receptor stimulation.

To test this possibility, we increased the amount of available G_q by stably transfecting a cDNA encoding recombinant G_q
into HEK293 cells already expressing the m3AChR. Carbachol, the m3AChR ligand, failed to elicit chemotaxis even in
the doubly transfected cells (Fig. 3A). The negative inference, that a G_q coupled receptor cannot elicit chemotaxis, was supported
by control observations (Fig. 3, B and C) indicating that recombinant G_q was indeed overexpressed and responsive to receptor
stimulation in these cells. Thus, expression of exogenous G_q allowed greater agonist-stimulated accumulation of phospha-
inositides than that observed either in cells expressing the m3AChR alone or in pertussis-toxin treated cells expressing
G_q and CXCR1 (Fig. 3B). Moreover, recombinant G_q and G_q were expressed to nearly identical extents in the two
types of cell (Fig. 3C), as indicated by immunoblots with mono-
clonal antibodies against epitopes inserted into both G_a proteins.

The failure of CXCR1 alone to mediate activation of phosphoinositide accumulation by IL-8 (Fig. 2C) is consistent with
results of a previous study (17), in which recombinant CXCR1 was found to activate some but not all members of the α_q
family, i.e. IL-8 stimulated phospholipase C in CXCR1-expressing COS-7 cells if they coexpressed α_14, α_15, or α_16, but did not do
so in cells expressing CXCR1 alone or in combination with α_1 or α_11. In the same study (17), CXCR1 mediated G_α- and
G_βγ-dependent activation of phospholipase C, but only in the presence of the β2 isoform of the phospholipase. In view of this
latter result, we suspect that HEK293 cells lack the β2 isofrom of the enzyme, because CXCR1 alone does not stimulate phos-
inositide accumulation in these cells (Fig. 2C).

DISCUSSION

Our experiments with HEK293 cells pose an intriguing two-
fold paradox. First, as reported earlier (9), liberation of G_βγ from G_αβγ is required for chemotaxis of these cells; nonetheless,
even though activation of any trimeric G protein releases G_βγ, receptors that activate G proteins other than G_3 do not
mediate chemotaxis. Second, even though receptors coupled to
G_q are required to mediate chemotaxis of these cells, signaling
by G_q itself is not required, at least in HEK293 cells. Here we
discuss four speculative ways to resolve these paradoxes: G_q-
coupled receptors may activate a specific subset of G_βγ iso-
forms, may generate a G_α_q-independent signal in addition to
G_βγ, may be susceptible to novel regulatory controls, or may
promote co-localization in the plasma membrane of appropriate
effectors with the G_βγ liberated by receptor activation. These
explanations are not mutually exclusive.

By choosing among polypeptides encoded by five G_β and 11
G_γ genes, mammalian cells could express many different G_βγ isoforms (6). Does a specific G_βγ isofrom mediate chemotaxis?
If so, the responsible G_βγ dimer must possess specificity not
only for a subset of G protein coupled receptors but also for
the specific downstream effector(s) of chemotaxis. In both respects
the evidence from other G protein-mediated signaling path-
ways is inconclusive. Receptors can select among polypeptides encoded by five G_β and 11 G_γ genes, mammalian cells could express many different G_βγ isoforms in vitro (6) and in regulating neuronal Ca_{2+} channels of intact cells (18, 19). Shared specificity for one G_βγ isofrom has not been reported, however, for any group of G protein-coupled receptors, including those that couple to G_i. With
respect to effectors, circumstantial evidence implicates G_α_i as an
essential component of G-mediated stimulation of phospholipase C_β in differentated HL60 cells (20), and γ_5 and γ_12 are
reported to colocalize in cultured cells with vinculin and F
actin, respectively (21). Nonetheless, experiments in several
laboratories have failed to show significant specificity of any
G_βγ dimer, except for the relative weakness of those containing
Gi-coupled receptor promotes liberation of G\textsubscript{a} arrestins, two potential candidates for generators of such a pathway (9), responses mediated by G\textsubscript{a} cyclase or stimulation of the mitogen-activated protein kinase Gi-coupled receptors and G protein subunits can serve as scaffolding proteins that associate with Gi-coupled receptors.

This fourth proposal for resolving the paradox raises an interesting question. Do all Gi-coupled receptors share the proposed ability of chemotactic receptors to organize signaling complexes that are required for chemotaxis? We and others have tested several Gi-coupled receptors, not previously identified as "professional" chemotactic receptors, for ability to mediate directional migration of cultured cells toward the appropriate ligand; the D2 dopamine receptor and the \(\mu\)- and \(\delta\)-opioid receptors do mediate chemotaxis, albeit not as efficiently as CXCR1 (9, 12). It is not clear whether this result can be generalized to include all, or even most, Gi-coupled receptors.

The paradox we have described is paralleled, nonetheless, by similar paradoxes in two other responses to agonists for Gi-coupled receptors: stimulation of the mitogen-activated protein kinase pathway and opening of K\textsubscript{i} channels. Even though G\textsubscript{\beta}\gamma (but not G\textsubscript{a}z-GTP) mediates both responses, neither response is elicited by the G\textsubscript{\beta}\gamma liberated by activating receptors that activate G proteins other than G\textsubscript{i} (32).\textsuperscript{2} Possible resolutions of these paradoxes include those we have outlined for resolving the paradox in chemotactic signaling.

Finally, our findings in HEK293 cells provide a starting point for dissecting the molecular basis of chemotactic signaling in neutrophils and other professionally chemotactic cells. Gi-coupled receptors and G protein subunits can serve as probes for identifying the critical but so far elusive effectors that harness the actin cytoskeleton to effect directional migration.

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