RESEARCH COMMUNICATION

Signalling through the leukotriene B_4 receptor involves both α_i and α_{16}, but not α_q or α_{11} G-protein subunits

Rémi GAUDREAU, Christian LE GOUILL, Salim MÉTAOUI, Stéphane LEMIRE, Jana STANKOVÀ and Marek ROLA-PLESZCZYNSKI[1]

Immunology Division, Department of Pediatrics, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, QC J1H 5N4 Canada

COS-7 cells transfected with the leukotriene (LT) B_4 receptor (BLTR) cDNA were unable to produce LTB_4-induced inositol phosphates (IPs) in spite of the presence of endogenous $G\alpha_i$, $G\alpha_q$ and $G\alpha_{11}$ proteins. Co-transfection of BLTR with $G\alpha_{16}$, however, resulted in high levels of IP production, which were 17-, 10- and 6-fold higher than with co-transfected $G\alpha_{11}$, $G\alpha_q$ and $G\alpha_{14}$, respectively. Co-transfection of BLTR with phospholipase C (PLC) β_2, on the other hand, resulted in efficient IP production and co-transfection of BLTR with both $G\alpha_{16}$ and PLCβ_2 resulted in a greater than additive response.

INTRODUCTION

Leukotriene (LT) B_4 is a powerful inflammatory mediator derived from lipoxygenation of arachidonic acid. It has been shown to be synthesized rapidly by phagocytic cells, principally neutrophils [1], upon challenge with a variety of stimuli. LTB_4 exerts a wide range of biological actions, such as chemotaxis, chemokinesis, neutrophil aggregation, degranulation and induction of cation fluxes (reviewed in [2]). We and others have also shown that LTB_4 modulates immune responses, including transcription of interleukin-2 receptor-α, interleukin-6 and c-*fos* [2–5].

The major pathway for LTB_4 signalling has been shown to involve *Bordetella pertussis* toxin (PTX)-sensitive G-protein(s) and to lead to activation of phosphoinositide (PI)-specific phospholipase C (PLC), release of inositol phosphates (IP) through PI hydrolysis and subsequent mobilization of intracellular Ca^{2+} [6–9]. Although sensitivity to PTX suggests involvement of $G\alpha_{i/0}$ subunits in LTB_4 signalling, several G-protein-coupled receptors for chemotactic factors (C5a, platelet-activating factor, C-C and CXC chemokines) have also been shown to couple to PTX-resistant α subunits of the G_q class ($G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$), which can activate PLCβ isoforms [10–15]. Expression of $G\alpha_q$ and $G\alpha_{11}$ proteins is ubiquitous, whereas that of $G\alpha_{14}$ and $G\alpha_{16}$ is more restricted. $G\alpha_{16}$ is detected predominantly in haematopoietic cells [16–19] and was reported recently in keratinocytes [20]; $G\alpha_{11}$ is expressed predominantly in spleen, lung and testis as well as in some lineages of haematopoietic cells [17]. Recently, Yokomizo et al. reported the cloning and sequencing of the receptor for LTB_4 (BLTR) from HL-60 cells differentiated into neutrophils [21]. They showed it to bind LTB_4 with high affinity and to transduce signals leading to chemotaxis. As reported for formyl-peptide and C5a receptors [22,23], signalling through BLTR was found to be predominantly, but not completely, PTX-sensitive, suggesting that G-protein subunits other than $G\alpha_{i/0}$ were also involved.

In the present study, we examined the G-protein-coupled signal transduction pathways for BLTR. To identify the G-proteins that coupled to BLTR, we used a co-transfection assay system in mammalian cells, and monitored the response to LTB_4 through PLC activation, which leads to IP production.

MATERIALS AND METHODS

Reagents

cDNAs encoding $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$ and PLCβ_2 were generous gifts from Dr. M. I. Simon (California Institute of Technology, Pasadena, CA, U.S.A.); the pJ3M expression vector [24] was a generous gift from Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA, U.S.A.). Other materials and their sources were as follows: lipofectamine and all culture media (Life Technologies Inc., Burlington, ON, Canada); PTX (Sigma Chemical Co., St.Louis, MO, U.S.A.); fetal bovine serum (Intergen, Purchase, NY, U.S.A.); *Pwo* polymerase (Boehringer Mannheim, Laval, QC, Canada); restriction endonuclease (Promega, Madison, WI, U.S.A.); T4 DNA ligase (Pharmacia Biotech Inc, Baie d'Urfé, Quebec, Canada); LTB_4 (Cayman Chemical, Ann Arbor, MI, U.S.A.); [^{3}H]*myo*-inositol (Amersham Canada Ltd, Oakville, ON, Canada); perchloric acid (VWR Canlab, Ville Mont-Royal, QC, Canada); FITC-conjugated goat anti-mouse antibody (BIO/CAN Scientific, Mississauga, ON, Canada).

BLTR cDNA cloning

The cDNA encoding human BLTR was cloned from the genomic DNA of Raji cells by PCR using primers (forward 5′-CGGATCCAACACTACATCTTCTGCAGCACCC-3′, and reverse 5′-GCGAATTCTAGTTCAGTTTAACTTGAG-3′) based on the published sequence (GenBank accession no. D89078). The resulting fragment was then digested with *Bam*HI–*Eco*RI restriction enzymes and subcloned into the *Bam*HI–*Eco*RI sites of the pJ3M expression vector. In this construction, the N-terminal

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; BLTR, leukotriene B_4 receptor; LT, leukotriene; IP, inositol phosphate; PTX, *Bordetella pertussis* toxin; PI, phosphoinositide; PLC, phospholipase C.

[1] To whom correspondence should be addressed (e-mail mrolaple@courrier.usherb.ca).

initiator methionine was replaced by the peptide sequence MEQKLISEEDLSRGSPG, resulting in a c-*myc* epitope-tagged BLTR coding sequence, in frame with the c-*myc* epitope. This sequence was verified by DNA sequencing (at the University of Calgary, Alberta, Canada).

COS cell expression vectors

To achieve similar expression levels, cDNAs corresponding to G-protein α-subunits (murine $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and human $G\alpha_{16}$) were all cloned into the cytomegalovirus vector pCIS [16,17,25]. PLCβ_2 and BLTR were expressed in the simian virus vector pMT2 and pJ3M [22] respectively.

Cell culture and transfection

COS-7 cells were maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM), containing 10 % fetal bovine serum. For transfection, COS-7 cells were plated in 30 mm dishes at a density of 2×10^5 cells/dish. The following day, plasmid cDNAs of BLTR, PLCβ_2 and/or different α subunits of the G_q family (0.2 μg of each cDNA, per dish) were premixed with 4 μl of lipofectamine and added to cells, followed by an incubation of 6 h. Cells were then washed and experiments were performed 48 h after transfection. The total amount of co-transfected DNA was kept constant in all co-transfection experiments by adding pcDNA3 vector DNA.

IP assay

Following transfection (24 h), COS-7 cells were labelled with [^{3}H]*myo*-inositol (3 μCi/ml) in DMEM without inositol for 18 h. In selected experiments, cells were also treated during this time (18 h) with 100 ng/ml of PTX. Medium was then removed and cells were incubated for 10 min with 10 mM LiCl in pre-warmed DMEM without inositol, containing 0.1 % BSA (w/v). Cells were then treated with medium or 100 nM LTB_4, for 30 min at 37 °C. After this incubation period, cells were lysed by addition of perchloric acid. Total [^{3}H]IP_{1-6} were extracted [26] and separated [27] on a Dowex AG1-X8 ($HCOO^-$ form) column (Bio-Rad Laboratories, Hercules, CA, U.S.A.) and then counted by liquid scintillation.

Assessment of the cell-surface expression of the receptor

Cell-surface expression of BLTR co-transfected with the cDNAs of different G_q family members in COS-7 cells was assessed by flow cytometry and by radioligand-binding assay. Flow cytometry was performed on 2.5×10^5 cells using anti-c-Myc antibodies (9E10 hybridoma, American Tissue Culture Collection, Bethesda, MD, U.S.A.), which recognized the c-Myc-tagged BLTR, followed by fluorescein isothiocyanate-conjugated goat anti-mouse antibody. All measures were performed on a FACScan flow cytometer (Becton-Dickinson).

Radioligand binding assay

LTB_4 binding was measured on intact cells. Cells (5×10^5) were harvested and resuspended in a volume of 100 μl of Hepes-Tyrode's buffer containing 0.1 % (w/v) BSA [28]. Binding reaction was performed in a final volume of 200 μl, in the presence of 0.25 nM [^{3}H]LTB_4, for 4 h at 12 °C. All points were in duplicate. The reaction was stopped by centrifugation and cell-associated radioactivity was measured by liquid scintillation.

RESULTS AND DISCUSSION

In the present study, we used a co-transfection system in COS-7 cells to define the capacity of BLTR to couple with the G_q class of G-proteins and signal through their activated Gα (α_q, α_{11}, α_{14}, α_{16}) subunits. COS-7 cells have been widely used to define interactions between GPCR, G-proteins and effectors, such as PLCβ isoforms. Endogenous expression of these proteins has also been determined. COS-7 cells contain endogenous $G\alpha_q$ and $G\alpha_{11}$, but no $G\alpha_{14}$ or $G\alpha_{16}$, $G\alpha_{i2}$, but no $G\alpha_o$, $G\alpha_{i1}$ or $G\alpha_{i3}$ [29], PLCβ_1 and PLCβ_3, but no PLCβ_2 [12,13]. Transfection of BLTR cDNA alone into COS-7 cells did not allow for LTB_4-dependent signalling, as measured by IP production. This suggested that BLTR could not activate endogenous PLCβ_1 or PLCβ_3 by coupling to endogenous $G\alpha_i$, $G\alpha_q$ or $G\alpha_{11}$. Even when the latter were over-expressed, LTB_4-induced IP production was minimal (10 ± 4 % and 6 ± 3 % of the maximal response, with BLTR and $G\alpha_{16}$, respectively; Table 1). Expression of $G\alpha_{14}$ with BLTR was only associated with a modest, ligand-induced IP production (15 ± 5 % of maximal response). In contrast, co-transfection of BLTR and $G\alpha_{16}$ cDNAs resulted in a 10-fold higher LTB_4-induced accumulation of IP (defined as 100 %: unstimulated, 568 ± 84 c.p.m.; LTB_4-stimulated, 6501 ± 340 c.p.m.; $P < 0.001$; Table 1). As expected, these G_q-mediated responses were not affected by PTX pretreatment (results not shown).

Responses to LTB_4 in leucocytes are predominantly PTX-sensitive [8,9], whereas the signal transduction pathways mediated by the α subunits of the G_q class are PTX-resistant [30]. Leucocytes have abundant $G\alpha_i$ proteins (mainly $G\alpha_{i2}$ and some $G\alpha_{i3}$) [31,32] and PLCβ_2 [33]. Moreover, levels of expression of $G\alpha_i$ increase during leucocyte differentiation [16]. $G\alpha_{16}$ is also predominantly expressed in haematopoietic cells, namely neutrophils, monocytes, lymphocytes and erythrocytes, as well as in various haematopoietic progenitor cells [16] and in keratinocytes [20]. Its expression decreases in human B cells when they mature [32] and in HL-60 promyeloid cells after differentiation into neutrophils, but not when they differentiate into monocytes [16,19]. In contrast, human T-cells express high levels of $G\alpha_{16}$, but low levels of $G\alpha_{i2}$ [34].

To test whether BLTR could couple to endogenous PTX-sensitive G-proteins in COS-7 cells to activate PLCβ_2, we co-transfected COS-7 cells with cDNAs encoding BLTR and PLCβ_2. The latter is not normally expressed in COS-7 cells, but is the major PLCβ isoform activated by interacting with the released

Table 1 Effects of G_q-class G-protein expression on BLTR-induced IP accumulation

COS-7 cells were transiently co-transfected with the cDNA (0.2 μg) encoding BLTR and the cDNA (0.2 μg) corresponding to $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$, $G\alpha_{16}$ or the pcDNA3 expression vector. Cells were stimulated with 100 nM LTB_4 for 30 min and IP were extracted as described in the Materials and methods section. Data represent IP accumulation over basal (non-stimulated) levels and are relative to those obtained in cells co-transfected with BLTR and $G\alpha_{16}$ cDNAs (defined as 100%). The results are the means $\pm$ S.D. of at least 3 experiments, each done in duplicate. Significant differences from BLTR + pcDNA3 are indicated as $P < 0.05$ (*) or $P < 0.001$ (**).

Transfection	IP accumulation (%)
BLTR	
+ pcDNA3	0.5 ± 1.0
+ $G\alpha_{11}$	5.7 ± 4.6
+ $G\alpha_q$	9.7 ± 2.6
+ $G\alpha_{14}$	15.0 ± 4.6*
+ $G\alpha_{16}$	100.0 ± 0.0**

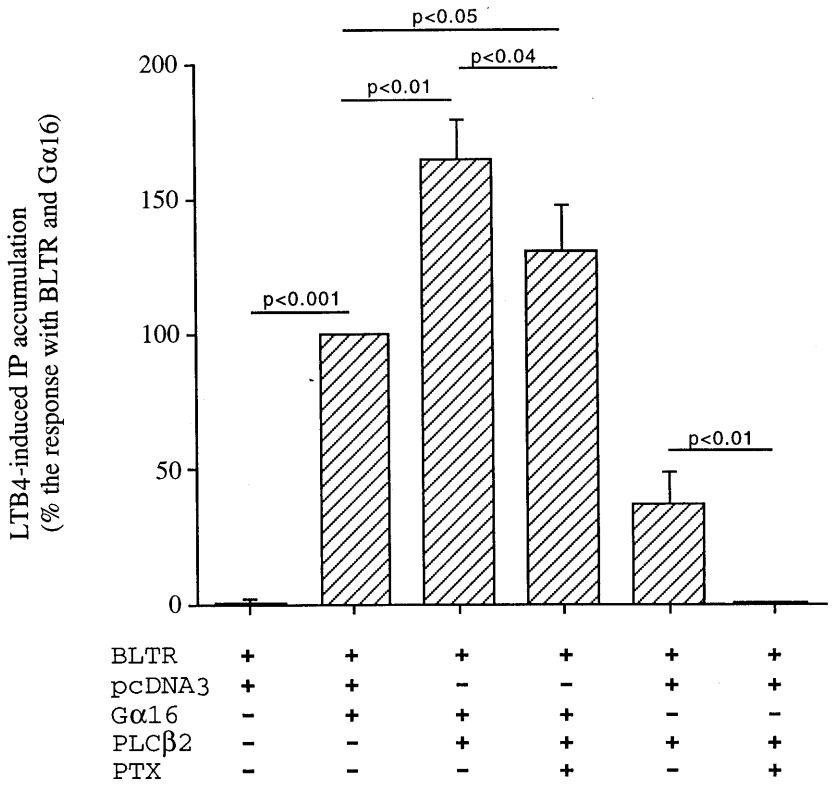

Figure 1 Effects of PTX on PLCβ_2 and Gα_{16}-dependent IP accumulation in response to LTB_4

COS-7 cells were transiently co-transfected with the cDNA (0.2 μg) encoding BLTR and the cDNA (0.2 μg) corresponding to Gα_{16}, PLCβ_2 or the pcDNA3 expression vector. Cells were incubated for 18 h before the day of the experiment in the presence or absence of PTX (100 ng/ml), then stimulated with 100 nM LTB_4 for 30 min, and IP were extracted as described in the Materials and methods section. Data represent IP accumulation over basal (non-stimulated) levels and are relative to those obtained in cells co-transfected with BLTR and Gα_{16} cDNAs and pcDNA3 vector (defined as 100%). The results are the means $\pm$ S.D. of at least 3 experiments, each done in duplicate. Statistical analysis was performed using the Student's *t* test.

G$\beta\gamma$ subunits from the PTX-sensitive G-proteins. As shown in Figure 1, BLTR was able to couple to a PTX-sensitive G protein to mediate a significant ($P < 0.01$) ligand-dependent activation of PLCβ_2. LTB_4-induced IP production, in these cells, represented $37 \pm 12\%$ of the levels measured in cells expressing Gα_{16}. Moreover, when cells were co-transfected with the triple combination of cDNAs encoding BLTR, Gα_{16} and PLCβ_2, a greater than additive ($159 \pm 10\%$ versus $128 \pm 8\%$; $P = 0.033$) effect was observed. In this context, pretreatment of cells with PTX resulted in a significant ($P < 0.04$), but partial, reduction of LTB_4-induced IP production, which remained higher than that of BLTR + Gα_{16}-transfected cells (Figure 1). Cell-surface expression of BLTR was not affected by co-transfection with Gα subunits and/or PLCβ_2, as assessed by flow cytometry. Similarly, specific binding of LTB_4 to BLTR-transfected cells was not affected by co-transfection with Gα subunits (results not shown).

BLTR can thus couple to and signal through endogenous PTX-sensitive G_i subunits. We presume that the interaction is with G_{i2} because it is the only known PTX-sensitive G-protein expressed in COS-7 cells [29]. No PTX-sensitive Gα subunits have been shown to activate PLCβ, whereas in a co-transfection system, G$\beta\gamma$ could activate PLC$\beta_{2,3}$ isoforms, but not PLC$\beta_{1,4}$ [15,29]. Although COS-7 cells contain endogenous PLCβ_3, the lack of response to LTB_4 in cells transfected with BLTR alone [or when PLCβ_3 was overexpressed by co-transfection (data not illustrated)] suggests that PLCβ_3 could not be activated by G$\beta\gamma$ subunits of endogenous G_{i2}, G_q, G_{11} or G_{14}. In smooth-muscle cells, however, which contain G_{i1} and G_0, G$\beta\gamma$ subunits were found to mediate activation of PLCβ_3 in response to somatostatin [35].

The physiological relevance of the activation pathways mediated by G_i and G_{16} remains to be elucidated, since both Gα subunits are found in haematopoietic cells, albeit in varying proportions. Our data indicate that, whereas PTX-sensitive activation of PLCβ_2, presumably via G_i-derived $\beta\gamma$ subunits, can mediate BLTR signalling in COS-7 cells, Gα_{16} mediates the most efficient coupling between BLTR and PI turnover in these cells. Whether Gα_{16} actually mediates LTB_4-dependent IP accumulation in cell types of haematopoietic origin remains to be resolved. Although responses to LTB_4 are predominantly PTX-sensitive in leucocytes [8,9], PTX-resistant effects of LTB_4 have been reported in endothelial cells [36]. Moreover, Yokomizo et al. [21] found Ca^{2+} mobilization in response to LTB_4 in BLTR-transfected Chinese hamster ovary cells to be mostly PTX-resistant, whereas chemotaxis was PTX-sensitive in these same cells.

Interestingly, our data indicate that both PTX-sensitive and PTX-resistant signalling pathways can be triggered concomitantly. Although it could have been expected that the added effect due to the presence of PLCβ_2 would be PTX-sensitive, most but not all of the additional response was prevented by pretreatment with PTX. Those results suggest that G$\beta\gamma$ subunits released from both Gα_i and Gα_{16}, and possibly Gα_{16} subunits themselves, participated in PLCβ_2 activation. This may be related to the fact that the activation sites on PLCβ isoforms by Gα_{16} and G$\beta\gamma$ subunits are different (reviewed in [37,38]). Gα_{16}

can be activated by a greater variety of GPCRs than can $G\alpha_q$, $G\alpha_{11}$ or $G\alpha_{14}$ [39], and our data indicate that this is also the case for BLTRs. Other receptors for chemotactic factors, such as formyl-Met-Leu-Phe receptor and C5aR, have also been shown to couple quite efficiently with $G\alpha_{16}$ [22,23].

Although *in vivo* effects of LTB_4 may be dependent on the level of expression of BLTR, they may also be modulated by the differential expression levels of the signalling effectors (G_i and G_{16}, and PLCβ proteins) in various cell populations and with regard to their stages of differentiation. Similarly, the differential expression of G$\beta\gamma$ proteins in various cell types would also contribute to signal transduction via BLTRs.

This work was supported by a grant from the Medical Research Council of Canada. The authors also wish to thank D. Gingras for excellent technical assistance.

REFERENCES

1 Borgeat, P. and Samuelson, B. (1979) J. Biol. Chem. **254**, 2643–2646
2 Rola-Pleszczynski, M. (1985) Immunol. Today **10**, 302–307
3 Stankova, J., Dupuis, G., Gagnon, N., Thivierge, M., Turcotte, S. and Rola-Pleszczynski, M. (1993) J. Immunol. **150**, 4041–4051
4 Rola-Pleszczynski, M. and Stankova, J. (1992) Blood **80**, 1004–1011
5 Stankova, J. and Rola-Pleszczynski, M. (1992) Biochem. J. **282**, 625–629
6 Lew, P. D., Dayer, J.-M., Wollheim, C. B. and Pozzan, T. (1984) FEBS Lett. **166**, 44–48
7 Andersson, T., Schlegel, W., Monod, A., Krause, K-H., Stendahl, O. and Lew, D. P. (1986) Biochem. J. **240**, 333–340
8 Shefcyk, J., Yassin, R., Volpi, M., Molski, T. F., Naccache, P. H., Monoz, J. J., Becker, E. L., Feinstein, M. B. and Sha'afi, R. I. (1985) Biochem. Biophys. Res. Commun. **126**, 1174–1181
9 Goldman, D. W., Chang, F.-H., Gifford, L. A., Goetzl, E. J. and Bourne, H. R. (1985) J. Exp. Med. **162**, 141–156
10 Smrcka, A. V., Hepler, J. R., Brown, K. O. and Sternweis, P. C. (1991) Science **251**, 804–807
11 Taylor, S. J., Chae, H. Z., Rhee, S. and Exton, J. (1991) Nature (London) **350**, 516–518
12 Wu, D. Q., Lee, C.-H., Rhee, S.-G. and Simon, M. I. (1992) J. Biol. Chem. **267**, 1811–1817
13 Wu, D., Katz, A. and Simon, M. I. (1993) Proc. Natl. Acad. Sci. U.S.A. **90**, 5297–5301
14 Lee, C.-H., Park, D., Wu, D., Rhee, S.-G. and Simon, M. I. (1992) J. Biol. Chem. **267**, 16044–16047
15 Jiang, H., Wu, D. and Simon, M. I. (1994) J. Biol. Chem. **269**, 7593–7596
16 Amatruda, III, T. T., Steele, D. A., Slepak, V. Z. and Simon, M. I. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5587–5591
17 Wilkie, T. M., Scherle, P. A., Strathmann, M. P., Slepak, V. Z. and Simon, M. I. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 10049–10053
18 Wilkie, T. M., Gilbert, D. J., Olsen, A. S., Chen, X. N., Amatruda, III, T. T., Korenberg, J. R., Trask, B. J., de Jong, P., Reed, R. R., Simon, M. I. et al. (1992) Nat. Genet. **1**, 85–91
19 Tenailleau, S., Corre, I. and Hermouet, S. (1997) Exp. Hematol. **25**, 927–934.
20 McAlpine Rock, B., Xin, L. and Wilcox, J. N. (1997) J. Invest. Dermatol. **109**, 645–649
21 Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y. and Shimizu, T. (1997) Nature (London) **387**, 620–624
22 Amatruda, III, T. T., Gerard, N. P., Gerard, C. and Simon, M. I. (1993) J. Biol. Chem. **268**, 10139–10144
23 Jiang, H., Kuang, Y., Wu, Y., Smrcka, A. and Simon, M. I. (1996) J. Biol. Chem **271**, 13430–13434
24 Sells, M. A. and Chernoff, J. (1995) Gene **152**, 187–189
25 Strathmann, M. and Simon, M. I. (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 9113–9117
26 Martin, T. F. J. (1983) J. Biol. Chem. **258**, 14816–14822
27 Berridge, M. T., Dawson, R. M. C., Downes, C. P., Hyslop, J. P. and Irvine, R. F. (1983) Biochem. J. **212**, 473–482
28 Honda, Z. I., Takano, T., Gotoh, Y., Nishida, E., Ito, K. and Shimizu, T. (1994) J. Biol. Chem. **269**, 2307–2315
29 Katz, A., Wu, D. and Simon, M. I. (1992) Nature (London) **360**, 686–689
30 Simon, M. I., Strathmann, M. P. and Gautam, N. (1991) Science **252**, 802–808
31 Murphy, P. M., Eide, B., Goldsmith, P., Brann, M., Gierschik, P., Spiegel, A. M. and Malech, H. L. (1987) FEBS Lett. **221**, 81–86
32 Goldsmith, P., Rossiter, K., Carter, A., Simond, W., Unson, C. G., Vinitsky, R. and Spiegel, A. M. (1988) J. Biol. Chem. **263**, 6476–6479
33 Kriz, R., Lin, L.-L., Sultzman, L., Ellist, C., Heldin, C.-H., Pawson, T. and Knopf, J. (1990) CIBA Found. Symp. **150**, 112–123
34 Mapara, M. Y., Bommert, K., Bargou, R. C., Leng, C., Beck, C., Ludwig, W. D., Gierschik, P. and Dörken, B. (1995) Blood **85**, 1836–1842
35 Murthy, K. S., Coy, D. H. and Makhlouf, G. M. (1996) J. Biol.Chem. **271**, 23458–23463
36 Palmblad, J., Lerner, R. and Larsson, S. H. (1994) J. Immunol. **152**, 262–269
37 Sternweis, P. C. (1994) Curr. Opin. Cell Biol. **6**, 403–406
38 Camps, M., Hou, C., Sidiropoulos, D., Stock, J. B., Jacobs, K.-H. and Gierschik, P. (1992) Eur. J. Biochem. **206**, 821–831
39 Offermanns, S. and Simon, M. I. (1995) J. Biol. Chem. **270**, 15175–15180

Received 30 June 1998/12 August 1998; accepted 18 August 1998