Volume 318, number 3, 277–281 FEBS 12163 March 1993

5-Lipoxygenase-activating protein is an arachidonate binding protein

Joseph A. Mancini[a], Mark Abramovitz[b], Martha E. Cox[b], Elizabeth Wong[a], Stella Charleson[a], Hélène Perrier[c], Zhaoyin Wang[c], Peptiboon Prasit[c] and Philip J. Vickers[a]

Departments of [a]Pharmacology, [b]Molecular Biology and [c]Medicinal Chemistry, Merck Frosst Centre for Therapeutic Research, Quebec H9R 4P8, Canada

Received 13 January 1993

5-Lipoxygenase-activating protein (FLAP) is an 18-kDa integral membrane protein which is essential for cellular leukotriene (LT) synthesis, and is the target of LT biosynthesis inhibitors. However, the mechanism by which FLAP activates 5-LO has not been determined. We have expressed high levels of human FLAP in *Spodoptera frugiperda* (Sf9) insect cells infected with recombinant baculovirus, and used this system to demonstrate that FLAP specifically binds [^{125}I]L-739,059, a novel photoaffinity analog of arachidonic acid. This binding is inhibited by both arachidonic acid and MK-886, an LT biosynthesis inhibitor which specifically interacts with FLAP. These studies suggest that FLAP may activate 5-LO by specifically binding arachidonic acid and transferring this substrate to the enzyme.

5-Lipoxygenase-activating protein; 5-Lipoxygenase; Arachidonic acid; Photoaffinity labeling; Baculovirus

1. INTRODUCTION

Leukotrienes are potent mediators of numerous biological processes, including chemotaxis, vascular permeability and smooth muscle contraction [1,2], and are implicated in the pathophysiology of various inflammatory and hypersensitivity disorders, including asthma and inflammatory bowel disease [3–5]. As both the soluble enzyme 5-lipoxygenase (5-LO) and the integral membrane protein 5-lipoxygenase-activating protein (FLAP) are essential for the cellular synthesis of leukotrienes [6], compounds which interact with these proteins are being developed as therapeutic agents.

In response to a variety of agents which stimulate cellular LT synthesis, including calcium ionophore A23187 [7], thapsigargin [8], IgE [9], and fMLP [10], 5-LO translocates from the soluble to a membrane compartment. The enzyme then catalyses both the oxygenation of arachidonic acid to 5-HPETE and the subsequent conversion of 5-HPETE to LTA_4 [11,12] which is the precursor of LTB_4 and the peptidoleukotrienes LTC_4, LTD_4 and LTE_4. Compounds which specifically bind to FLAP, such as MK-886 [13,14] and MK-0591 [15] inhibit this membrane association of 5-LO. While membrane association of 5-LO may be required for the efficient utilization of arachidonic acid, which is released from membrane phospholipids by phospholipases, the critical role that FLAP plays in this process has not been determined.

To assess whether FLAP specifically binds a substrate of 5-LO, we synthesized [^{125}I]L-739,059, a radioiodinated photoaffinity analog of arachidonic acid. This ligand specifically binds to human FLAP expressed in *Spodoptera frugiperda* (Sf9) insect cells using recombinant baculovirus, with this binding being inhibited both by arachidonic acid and MK-886. These studies suggest that FLAP may activate 5-LO by specifically binding arachidonate and transferring this substrate to the enzyme for the concerted synthesis of LTA_4.

2. MATERIALS AND METHODS

2.1. *Chemicals and reagents*

Nucleotides and dNTPs were obtained from Boehringer Mannheim

Correspondence address: P.J. Vickers, Department of Pharmacology, Merck Frosst Centre for Therapeutic Research, P.O. Box 1005, Pointe Claire-Dorval, Quebec H9R 4P8, Canada. Fax: (1) (514) 428-8615.

Abbreviations: 5-HPETE, (5*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; LTA_4, 5,6-oxido-7,9,11,14-eicosatetraenoic acid; LTB_4, (5*S*,12*R*)-dihydroxy-6,8,10,14-eicosatetraenoic acid; LTC_4, (5*S*)-hydroxy-(6*R*)-*S*-glutathionyl-7,9,-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; LTD_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinylglycyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; LTE_4, (5*S*)-hydroxy-(6*R*)-*S*-cysteinyl-7,9-(*trans*)-11,14-(*cis*)-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; FLAP, 5-lipoxygenase-activating protein; MK-886, 3-[1-(*p*-chlorophenyl)-5-isopropyl-3-tert-butylthio-1*H*-indol-2-yl]-2,2-dimethylpropanoic acid (formerly designated L-663,536); L-691,831, ((1-(4-iodobenzyl)-3-(3,3-dimethyl-1-oxo-1-butyl)-5-quinol-2-ylmethoxy)-indol-2-yl)-2,2-dimethyl propionic acid; L-691,678, 1*H*-indole-2-propanoic acid, 3-[(4-azidophenyl)sulfonyl]-2-[(4-iodophenyl)methyl]-α,α-dimethyl-5-(2-quinolinylmethoxy)-; DMSO, dimethyl sulphoxide; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; EDTA, ethylenediamine tetraacetic acid; IC_{50}, concentration required for 50% inhibition.

(Montreal, Quebec, Canada). Restriction endonucleases, DNA polymerase I, Klenow fragment, T4 DNA ligase and T7 sequencing kit were obtained from Pharmacia (Montreal, Quebec, Canada). Radioisotopes ^{125}I-labeled Protein A (10 mCi/mg) and [α-^{32}P]dCTP (3,000 Ci/mmol) were supplied by NEN-DuPont (Burlington, Ontario, Canada). The synthesis of [^{125}I]L-739,059 will be described elsewhere.

2.2. *Cells and viruses*

Escherichia coli DH5α was obtained from Gibco/BRL and transformed according to published methods [16]. Sf9 insect cells were cultured in Grace's complete medium (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 50 μg of gentamicin sulfate per ml, and 2.5 μg of amphotericin B (Fungizone) per ml in either Corning T flasks (Richmond Hill, Ontario, Canada) or spinner flasks (Bellco Glass, Inc., Vineland, NJ) at 27°C following the procedures of Summers and Smith [17]. Sf9 insect cells and the pETL transfer vector were kindly provided by Dr. C.D. Richardson, Biotechnology Research Institute, Montreal, Quebec. Cell counts and cell viability were determined in 0.2% Trypan blue using a hemocytometer.

2.3. *Construction of recombinant transfer vector pETL-FLAP*

The human FLAP cDNA [6] was subcloned from the vector pBShFLAP [18] into the pETL transfer vector. Miniprep DNA from bacterial transformants was isolated, and the proper orientation of the inserted fragment with respect to the polyhedrin promoter was confirmed by restriction digest and sequence analysis. The 11-kb vector, pETL-FLAP, was purified using Magic Miniprep (Promega, Madison, WI) and subsequently used for co-transfection with wild type baculovirus DNA.

2.4. *DNA transfections and plaque assays*

Linearized wild type AcMNPV DNA (1 μg) (Invitrogen, San Diego, CA) was mixed with pETL-FLAP (2 μg) and co-transfected into Sf9 cells by the calcium phosphate method [17]. After 4 days, cellular debris was removed by centrifugation at 1,000 $\times$ g for 5 min and the supernatant was used as the source of recombinant virus for the initial plaque assay. Plaque assays were carried out in culture dishes (100 $\times$ 15 mm) as previously described [19]. From this initial plaque assay, 10 blue plaques were subjected to dot blot analysis as previously described [19] and probed with a fragment of the human FLAP cDNA. Nine of the ten plaques which expressed β-galactosidase activity also contained the FLAP cDNA (data not shown). Pure recombinant virus was isolated from two of the FLAP-positive plaques. One of these isolates, designated rvFLAP600-9, was used to infect Sf9 cells (1.0–2.0 $\times$ 10^6 cells/ml) at a multiplicity of infection of 10.

2.5. *Subcellular fractionation*

Human polymorphonuclear leukocytes were prepared from buffy coat concentrates by dextran sedimentation and hypotonic lysis of contaminating red blood cells as previously described [20]. These membranes have previously been shown to contain FLAP [21]. Sf9 cells were harvested by centrifugation (300 $\times$ g, 5 min, room temperature) and washed with Dulbecco's phosphate-buffered saline (D-PBS; Gibco/BRL). Cell pellets were then sonicated in 15 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM phenylmethylsulfonyl fluoride at 4°C, and the 100,000 $\times$ g membrane fraction prepared as previously described [18].

2.6. *Protein immunoblotting*

Protein samples were separated on 8–16% polyacrylamide gels (Novex, San Diego, CA) in the presence of SDS and transferred to nitrocellulose overnight using the Western Transfer apparatus (Novex) according to the manufacturer's instructions. Immunoblot analysis was performed as previously described [22], using a 1:200 dilution of antiserum and [^{125}I]Protein A (NEN-DuPont) as the detection system. The rabbit polyclonal antiserum used for these studies is designated H5 and recognizes amino acid residues 41–52 of FLAP [23]. Protein concentrations were determined in microtitre plates using Coomassie protein assay reagent (Pierce, Rockford, IL) according to the manufacturer's instructions, with bovine serum albumin as a standard.

2.7. *Radioligand binding assays*

Filter binding assays were performed with the quindole LT biosynthesis inhibitor [^{125}I]L-691,831 as previously described (Charleson et al., 1992), using membrane preparations from Sf9 cells. Specific binding in this assay is defined as the difference between the binding of [^{125}I]L-691,831 to membranes in the absence and presence of MK-886 at a concentration of 10 μM.

2.8. *Photoaffinity labeling and immunoprecipitation*

Photoaffinity labeling and immunoprecipitation of photoaffinity labeled protein were performed as previously described [23] using the quindole LT biosynthesis inhibitor [^{125}I]L691,678, or [^{125}I]L-739,059, a photoaffinity analogue of arachidonic acid. The rabbit polyclonal antiserum used for these studies is designated 709, and recognizes amino acid residues 1–39 of FLAP [21].

3. RESULTS

3.1. *Expression of FLAP in Sf9 insect cells*

Immunoblot analysis (Fig. 1) shows that while FLAP was undetectable in uninfected Sf9 cells or Sf9 cells infected with wild-type baculovirus, high levels of human FLAP were present in 100,000 $\times$ g membrane preparations of Sf9 cells following infection with

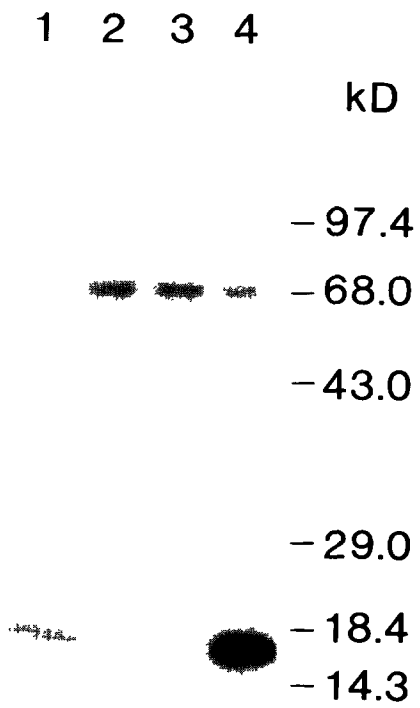

Fig. 1. Expression of human FLAP in Sf9 cells. Expression of FLAP was assessed in 6 μg of a 100,000 $\times$ g membrane preparation from human leukocytes (lane 1) or 20 μg of 100,000 $\times$ g membrane preparations from uninfected Sf9 cells (lane 2), Sf9 cells infected with wild-type baculovirus for 48 h (lane 3), or Sf9 cells infected with rvFLAP600-9 for 48 h (lane 4) by immunoblot analysis as described in section 2. Blots were exposed to X-ray film at −80°C for 60 min. The migration positions of molecular weight markers are indicated.

rvFLAP600-9 for 48 h. FLAP was undetectable in the 100,000 × *g* supernatant fraction of infected cells, suggesting that the protein is efficiently transferred to a membrane fraction when expressed in Sf9 insect cells. The maximal levels of FLAP obtained in membrane preparations of Sf9 cells were approximately 20-fold higher than those of human leukocyte preparations.

We next addressed whether FLAP expressed in Sf9 cells specifically binds LT biosynthesis inhibitors. A radioligand binding assay for FLAP utilizing the inhibitor [^{125}I]L-691,831 has recently been developed [24]. Using this assay, analysis of membrane preparations from Sf9 cells containing FLAP demonstrated saturable ligand binding, with specific binding representing at least 90% of total ligand binding at all protein concentrations analyzed (Fig. 2A). No specific binding of [^{125}I]L-691,831 was detectable using membrane preparations from uninfected Sf9 cells or to membrane preparations of cells infected with wild-type baculovirus. To confirm that FLAP expressed in Sf9 cells directly binds LT biosynthesis inhibitors, membrane preparations from infected cells were labeled with the radioiodinated photoaffinity ligand [^{125}I]L-691,678, an LT biosynthesis inhibitor which has previously been shown to covalently attach to FLAP [23]. Following photoaffinity labeling, proteins were immunoprecipitated with an anti-FLAP antiserum and analyzed by SDS-PAGE (Fig. 2B). A single radiolabeled protein, corresponding to the migration position of FLAP, was immunoprecipitated. Labeling of this protein was inhibited by preincubation with MK-886.

3.2. *Specific binding of arachidonic acid to FLAP*

When membrane preparations of Sf9 cells infected with rvFLAP600-9 and expressing high levels of FLAP were labeled with [^{125}I]L-739,059, a photoaffinity analog of arachidonic acid, and analyzed by SDS-PAGE, a radiolabeled species corresponding to the migration position of FLAP was observed (Fig. 3). This radiolabeled protein was not observed using membrane preparations from uninfected Sf9 cells or membrane preparations from infected cells which were boiled for 5 min prior to photolysis (data not shown). Photoaffinity labeling of this protein was inhibited by arachidonic acid in a concentration-dependent manner, with an IC_{50} of 10–20 μM. The identity of this radiolabeled species as FLAP was confirmed by immunoprecipitation using a FLAP-specific antiserum (Fig. 3). Photoaffinity labeling of FLAP with [^{125}I]L-739,059 was also competed by MK-886, but not by L-685,079 or L-671,480 (Fig. 3), indole and quinoline compounds which are only weak inhibitors of LT biosynthesis in human leukocytes [23].

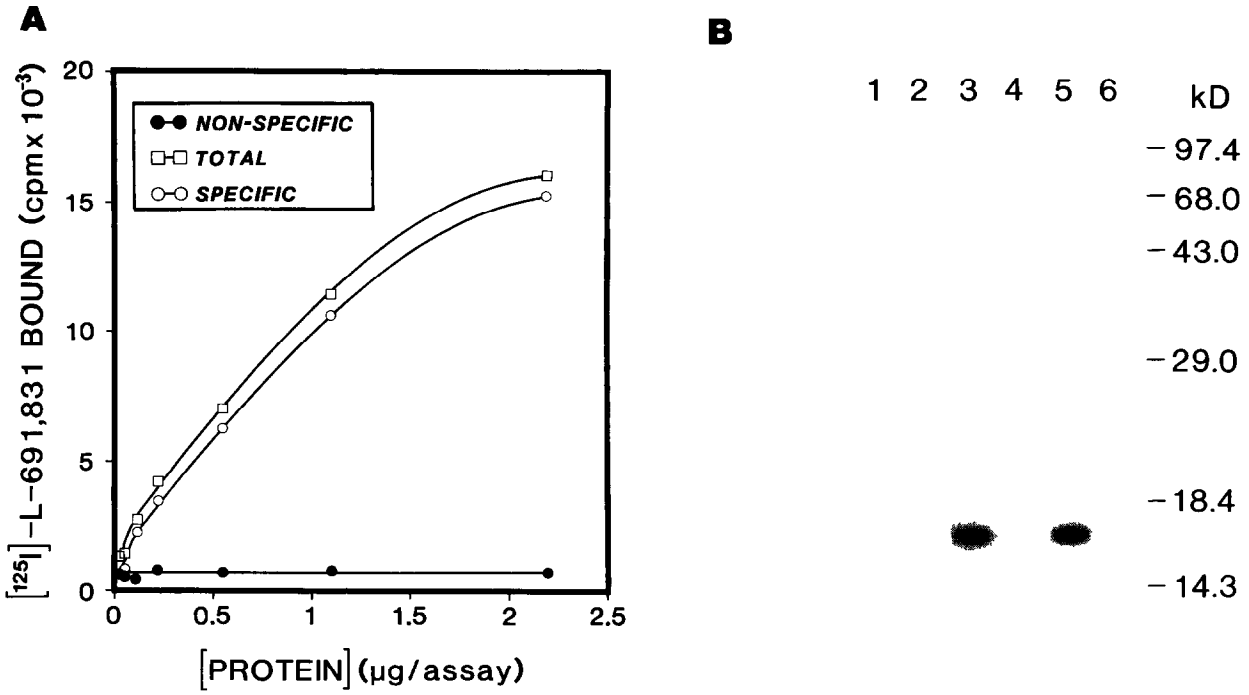

Fig. 2. Binding of LT biosynthesis inhibitors to FLAP. Following infection of Sf9 cells with rvFLAP600-9 for 48 h, the binding of LT biosynthesis inhibitors to 100,000 × *g* membrane preparations was analyzed by radioligand binding analysis (A) or photoaffinity labeling (B). (A) Radioligand binding analysis of the indicated concentrations of a membrane preparation from Sf9 cells infected with rvFLAP600-9 for 48 h were performed using [^{125}I]L-691,831 as described in section 2. (B) Membrane preparations from Sf9 cells infected with wild-type baculovirus for 48 h (lanes 1 and 2), Sf9 cells infected with rvFLAP600-9 for 48 h (lanes 3 and 4) or human leukocytes (lanes 5 and 6) were photoaffinity labeled with [^{125}I]L-691,678 and immunoprecipitated with antiserum 709 as described in section 2. Immunoprecipitation was performed following photoaffinity labeling in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 1 μM MK-886. Samples were then separated by 13.5% SDS-PAGE and dried gels exposed to X-ray film for 3 days at −80°C. The migration positions of molecular weight markers are indicated.

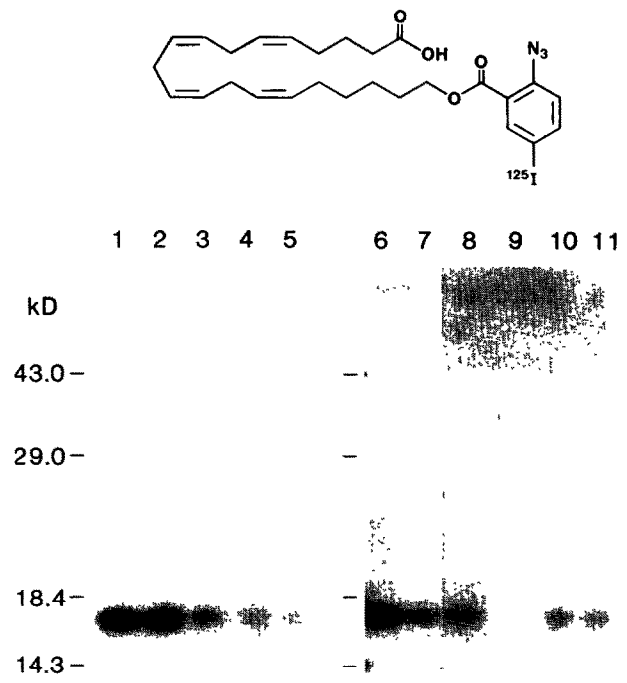

Fig. 3. Photoaffinity labeling of FLAP with [^{125}I]L-739,059. The 100,000 × g membrane fraction from Sf9 cells infected with rvFLAP600-9 for 48 h was photoaffinity-labeled with [^{125}I]L-739,059 (500–1,000 Ci/mmol) as described in section 2, using 7.5×10^5 cpm (lanes 1–7) or 3×10^5 cpm (lanes 8–11). Photoaffinity labeling was performed in the presence of DMSO vehicle (lanes 1, 6 and 8), arachidonic acid at a concentration of 1 μM (lane 2), 10 μM (lane 3), 25 μM (lane 4), or 50 μM (lane 5), 1 μM MK-886 (lanes 7 and 9), 1 μM L-685,079 (lane 10) or 1 μM L-671,480 (lane 11). For samples 1–5 and 8–11, 50 μg of protein was labeled. For samples 6 and 7, 300 μg of protein was labeled and these samples were immunoprecipitated using antiserum 709, as described in section 2. Samples were separated by 13.5% SDS-PAGE, and dried gels exposed to X-ray film for 3 days at −80°C. The migration positions of molecular weight markers are indicated. The structure of [^{125}I]L-739,059 is shown.

4. DISCUSSION

In recent years it has been demonstrated that FLAP plays an essential role in the cellular synthesis of LTs and represents a suitable target for LT biosynthesis inhibitors. However, the specific role that FLAP plays in LT biosynthesis has not been determined. In the present study we describe the first high level expression of FLAP, using the recombinant baculovirus rvFLAP600-9 to generate levels of human FLAP in Sf9 insect cells which are approximately 20-fold higher than those present in human leukocytes. FLAP which is incorporated into membranes of Sf9 cells can specifically bind LT biosynthesis inhibitors, as determined by a noncovalent radioligand binding assay as well as by immunoprecipitation of FLAP to which the radioiodinated photoaffinity ligand [^{125}I]L-691,678 is bound. Thus, the Sf9 expression system provides a rich source of FLAP for studies to analyze both the function and inhibitor binding characteristics of the protein.

We have used Sf9 cell membranes containing human FLAP in conjunction with [^{125}I]L-739,059, a novel photoaffinity analog of arachidonate, to demonstrate that FLAP specifically binds arachidonic acid, and that this binding is competed by the LT biosynthesis inhibitor MK-886. Interestingly, the IC_{50} for inhibition of [^{125}I]L-739,059 binding to FLAP by arachidonic acid (10–20 μM) is similar to the K_m for arachidonic acid determined for 5-LO from porcine [25] and human [26] leukocytes. The demonstration that FLAP specifically binds arachidonic acid suggests that FLAP may activate 5-LO by binding and transferring this substrate to the enzyme for the concerted synthesis of LTA_4. Consistent with this hypothesis, exogenous arachidonate increases levels of LT synthesis in human leukocytes, and this synthesis remains FLAP-dependent as it is inhibited by MK-886 [27].

Acknowledgements: The authors would like to thank Dr. D. Miller and E. Rands for providing antiserum 709, and Drs. J. Evans and A.W. Ford-Hutchinson for helpful discussions.

REFERENCES

[1] Samuelsson, B., Dahlen, S.E., Lindgren, J.A., Rouzer, C.A. and Serhan, C.N. (1987) Science 237, 1171–1176.
[2] Jones, T.R., Letts, G., Chan, C. and Davies, P., in: Leukotrienes and Lipoxygenases, Vol. 2 (J. Rokach, Ed.), Elsevier, Amsterdam, 1989, pp. 309–403.
[3] Ford-Hutchinson, A.W., in: Leukotrienes and Lipoxygenases, Vol. 2 (J. Rokach, Ed.), Elsevier, Amsterdam, 1989, pp. 405–425.
[4] Lewis, R.A., Austen, K.F. and Soberman, R.J. (1990) N. Engl. J. Med. 323, 645–655.
[5] Piacentini, G.L. and Kaliner, M.A. (1991) Am. Rev. Respir. Dis. 143, S96–S99.
[6] Dixon, R.A.F., Diehl, R.E., Opas, E., Rands, E., Vickers, P.J., Evans, J.F., Gillard, J.W. and Miller, D.K. (1990) Nature 343, 282–284.
[7] Rouzer, C.A. and Kargman, S. (1988) J. Biol. Chem. 263, 10980–10988.
[8] Wong, A., Cook, M.N., Foley, J.F., Sarau, H.M., Marshall, P. and Hwang, S.M. (1991) Biochemistry 30, 9346–9354.
[9] Wong, A., Cook, M.N., Hwang, S.M., Sarau, H.M., Foley, J.F. and Crooke, S.T. (1992) Biochemistry 31, 4046–4053.
[10] Kargman, S., Prasit, P. and Evans, J.F. (1991) J. Biol. Chem. 266, 23745–23752.
[11] Rouzer, C.A., Matsumoto, T. and Samuelsson, B. (1986) Proc. Natl. Acad. Sci. USA 83, 857–861.
[12] Shimizu, T., Izumi, T., Seyama, Y., Tadokor, K., Radmark, O. and Samuelsson, B. (1986) Proc. Natl. Acad. Sci. USA 83, 4175–4179.
[13] Gillard, J.W., Ford-Hutchinson, A.W., Chan, C., Charleson, S., Denis, D., Foster, A., Fortin, R., Léger, S., McFarlane, C.S., Morton, H., Piechuta, H., Riendeau, D., Rouzer, C.A., Rokach, J., Young, R., MacIntyre, D.E., Peterson, L., Bach, T., Ermann, G., Hopple, S., Humes, J., Hupe, L., Luell, S., Metzger, J., Meurer, R., Miller, D.K., Opas, E. and Pacholok, S. (1989) Can. J. Physiol. Pharmacol. 67, 456–464.
[14] Rouzer, C.A., Ford-Hutchinson, A.W., Morton, H.E. and Gillard, J.W. (1990) J. Biol. Chem. 265, 1436–1442.
[15] Brideau, C., Chan, C., Charleson, S., Denis, D., Evans, J.F., Ford-Hutchinson, A.W., Fortin, R., Gillard, J.W., Guay, J.,

Guévremont, D., Hutchinson, J., Jones, T., Léger, S., Mancini, J.A., McFarlane, C., Pickett, C., Piechuta, H., Prasit, P., Riendeau, D., Rouzer, C.A., Tagari, P., Vickers, P.J., Young, R.N. and Abraham, W.M. (1992) Can. J. Physiol. Pharmacol. 70, 799–807.

[16] Hanahan, D., in: DNA Cloning: A Practical Approach (D.E. Glover, Ed.), IRL Press, Arlington, VA, 1985, pp. 109–135.

[17] Summers, M.D. and Smith, G.E., in: A Manual for Baculovirus Vectors and Insect Cell Culture Procedures (Bulletin No. 1555), Texas Agriculture Experimental Station, College Station, TX, 1987, pp. 10–39.

[18] Vickers, P.J., Adam, M., Charleson, S., Coppolino, M.G., Evans, J.F. and Mancini, J.A. (1992) Mol. Pharmacol. 42, 94–102.

[19] Vialard, J., Lalumiere, M., Vernet, T., Briedis, D., Alkhatib, G., Henning, D., Lewis, D. and Richardson, C. (1990) J. Virol. 64, 37–50.

[20] Rouzer, C.A. and Samuelsson, B. (1985) Proc. Natl. Acad. Sci. USA 82, 6040–6044.

[21] Miller, D.K., Gillard, J.W., Vickers, P.J., Sadowski, S., Léveillé, C., Mancini, J.A., Charleson, P., Dixon, R.A.F., Ford-Hutchinson, A.W., Fortin, R., Gauthier, J.Y., Rodkey, J., Rosen, R., Rouzer, C., Sigal, I.S., Strader, C.D. and Evans, J.F. (1990) Nature 343, 278–281.

[22] Reid, G.K., Kargman, S., Vickers, P.J., Mancini, J.A., Léveillé, C., Éthier, D., Miller, D.K., Gillard, J.W., Dixon, R.A.F. and Evans, J.F. (1990) J. Biol. Chem. 265, 19818–19823.

[23] Mancini, J.A., Prasit, P., Coppolino, M.G., Charleson, P., Léger, S., Evans, J.F., Gillard, J.W. and Vickers, P.J. (1992) Mol. Pharmacol. 41, 267–272.

[24] Charleson, S., Prasit, P., Léger, S., Gillard, J.W., Vickers, P.J., Mancini, J.A., Charleson, P., Guay, J., Ford-Hutchinson, A.W. and Evans, J.F. (1992) Mol. Pharmacol. 41, 873–879.

[25] Ueda, N., Kaneko, S., Yoshimoto, T. and Yamomoto, S. (1986) J. Biol. Chem. 261, 7982–7988.

[26] Lewis, R.A. and Austen, K.F. (1984) J. Clin. Invest. 73, 889–897.

[27] Ménard, L., Pilote, S., Naccache, P.H., Laviolette, M. and Borgeat, P. (1990) Br. J. Pharmacol. 100, 15–20.