Granulocyte/Macrophage Colony-stimulating Factor Stimulates the Expression of the 5-Lipoxygenase–activating Protein (FLAP) in Human Neutrophils

By Marc Pouliot, Patrick P. McDonald, Pierre Borgeat, and Shaun R. McColl

From Le Centre de Recherche en Inflammation, Immunologie, et Rhumatologie, Centre de Recherche du CHUL, Université Laval, Québec, Canada G1V 4G2

Summary

The synthesis of leukotrienes in human blood neutrophils chiefly relies on the activity of two enzymes, phospholipase A_2 and 5-lipoxygenase (5-LO). In turn, the activation of the 5-LO requires the participation of a recently characterized membrane-bound protein, the 5-LO–activating protein (FLAP). In this study, we have investigated conditions under which FLAP expression in neutrophils may be modulated. Of several cytokines tested, only granulocyte/macrophage colony-stimulating factor (GM-CSF) (and to a lesser extent tumor necrosis factor α) significantly increased expression of FLAP. GM-CSF increased FLAP mRNA steady-state levels in a time- and dose-dependent manner. The stimulatory effect of GM-CSF on FLAP mRNA was inhibited by prior treatment of the cells with the transcription inhibitor, actinomycin D, and pretreatment of the cells with the protein synthesis inhibitor, cycloheximide, failed to prevent the increase in FLAP mRNA induced by GM-CSF. The accumulation of newly synthesized FLAP, as determined by immunoprecipitation after incorporation of ^{35}S-labeled amino acids, was also increased after incubation of neutrophils with GM-CSF. In addition, the total level of FLAP protein was increased in GM-CSF–treated neutrophils, as determined by two-dimensional gel electrophoresis, followed by Western blot. GM-CSF did not alter the stability of the FLAP protein, indicating that the effect of GM-CSF on FLAP accumulation was the consequence of increased de novo synthesis as opposed to decreased degradation of FLAP. Finally, incubation of neutrophils with the synthetic glucocorticoid dexamethasone directly stimulated the upregulation of FLAP mRNA and protein, and enhanced the effect of GM-CSF. Taken together, these data demonstrate that FLAP expression may be upmodulated after appropriate stimulation of neutrophils. The increase in FLAP expression induced by GM-CSF in inflammatory conditions could confer upon neutrophils a prolonged capacity to synthesize leukotrienes.

The leukotrienes are a family of lipid mediators of inflammation and allergy that exhibit a diverse range of potent biological effects (1, 2). Leukotriene (LT)[1] B_4 is a stereospecific activator of phagocyte function, stimulating chemotaxis, degranulation, adhesion, and aggregation (1, 2), whereas the cysteinyl-leukotrienes, LTC_4, LTD_4, and LTE_4 (formerly known as the slow-reacting substance of anaphylaxis), potently stimulate broncho-constriction and are vasoactive (1, 2). Because of the likelihood that LTB_4 is an important inflammatory mediator, and that the cysteinyl-leukotrienes play a key role in asthma and allergic reactions, considerable effort is still being placed in determining the mechanism by which their synthesis is regulated.

The conversion of free arachidonic acid to leukotrienes in phagocytes is controlled by the key enzyme, arachidonate 5-lipoxygenase (5-LO) (1–3). Recent studies have revealed new insights into the mechanisms by which the activation of this enzyme is regulated in intact cells. In particular, it was recently discovered that for leukotriene synthesis to occur, it is necessary for the 5-LO to be translocated from the cytosol to membrane structures (4, 5). A second major breakthrough occurred with the discovery of a membrane-bound protein, termed 5-LO–activating protein (FLAP) (6, 7). Several lines of evidence indicate that leukotriene synthesis will not occur in intact cells unless FLAP is present (6, 8). Studies involving cotransfection of cells with FLAP and 5-LO indicate that both

[1] *Abbreviations used in this paper:* AD, actinomycin D; CX, cycloheximide; FLAP, 5-lipoxygenase–activating protein; 5-LO, arachidonate 5-lipoxygenase; LT, leukotriene; rh, recombinant human; TBS, Tris-buffered saline.

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of these proteins are essential for leukotriene synthesis (6, 8). In addition, the indole derivative, MK 886, which binds to FLAP and prevents the translocation of the 5-LO, completely inhibits leukotriene synthesis in intact cells (9). The results of these studies all suggest that FLAP plays a pivotal role in leukotriene synthesis in intact cells.

FLAP expression in leukotriene-producing cells is thus critical for leukotriene synthesis to occur. However, it is not yet known whether the level of FLAP protein can be modulated in terminally differentiated phagocytes such as neutrophils. The 5'-regulatory region of the FLAP gene has recently been cloned and sequenced (10), and shown to contain several potential *cis*-regulatory regions characteristic of genes that are regulated at the transcriptional level. These latter results support the contention that the FLAP gene may be modulated in mature phagocytes. Since recent studies in our laboratory have shown that neutrophils are capable of significant RNA and protein synthesis in response to activation by cytokines including GM-CSF and TNF-α (11, 12), we have investigated the effect of various cytokines on FLAP gene expression in human neutrophils. Our data indicated that GM-CSF (and to a lesser extent, TNF-α), upregulates FLAP expression in a dose- and time-dependent fashion. Incubation of neutrophils with the synthetic glucocorticoid, dexamethasone, also led to an increase in FLAP expression.

Materials and Methods

Chemicals. The 0.84-kb FLAP cDNA probe cloned into the EcoRI site of the Stratagene SK vector (Stratagene, La Jolla, CA) and the rabbit polyclonal FLAP antibody were generously supplied by Dr. Jillian Evans and Dr. Philip Vickers of the Merck-Frosst Center for Therapeutic Research (Pointe-Claire, Québec, Canada). ^{35}S-labeled methionine and cysteine, horseradish peroxidase–linked donkey anti–rabbit antibody, and the enhanced chemoluminescence (ECL) detection kit were purchased from Amersham Canada (Oakville, Ontario, Canada). Immobilon-P and Hybond-N blotting membranes were from Millipore Corp. (Mississauga, Ontario, Canada). Protein-G Sepharose 4FF was purchased from Pharmacia LKB (Montréal, Québec, Canada). RPMI 1640 culture medium and FCS were purchased from GIBCO BRL (Burlington, Ontario, Canada). Recombinant human (rh) GM-CSF expressed in *Escherichia coli*, rhIL-3 and rhIL-6 were generous gifts from the Genetics Institute (Cambridge, MA). TNF-α was a generous gift from Knoll Pharmaceuticals (Whippany, NJ). Interferon-γ was obtained from Genentech Inc. (South San Francisco, CA). Cytokines were kept at −20°C as stock solutions in PBS containing 0.01% BSA. Tests on these solutions using the *Limulus amoebocyte* assay for lipopolysaccharide were negative. Hepes, Pipes, EGTA, EDTA, ATP, NP-40, Tween-20, dexamethasone, cycloheximide (CX), and actinomycin D (AD) were purchased from the Sigma Chemical Co., (St. Louis, MO). CX was dissolved in DMSO at a concentration of 10 mg/ml and stored at 4°C. AD was dissolved in distilled ethanol and kept at 4°C. The concentration of the stock solution was measured by spectrophotometry.

Cell Purification. Peripheral blood was obtained from healthy donors using either EDTA or heparin as an anticoagulant, and neutrophils were isolated essentially as described (11–13). The final cell suspensions contained <1% monocytes as determined by esterase staining, and the viability was >99%, as determined by Trypan Blue exclusion.

Northern Blot. Total RNA was purified as previously described (12). Briefly, RNA was isolated using a solution of guanidium isothiocyanate and centrifugation through a cesium chloride gradient. The RNA (15 μg/well) was then run on a 1% agarose gel containing formaldehyde, and transferred to Hybond-N membranes using a VacuGene apparatus (Pharmacia LKB). Integrity of the RNA and equal loading were verified by ethidium bromide staining and hybridization with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or 28S ribosomal RNA probes. The membranes were then hybridized with a FLAP cDNA probe, labeled with [^{32}P]CTP using a random primer kit (Amersham Canada).

Metabolic Labeling and FLAP Immunoprecipitation. Neutrophils were suspended at a cell density of 10^8/ml in methionine- and cysteine-free RPMI 1640 supplemented with 0.1% FCS and containing 200 μCi/ml of both [^{35}S]methionine and [^{35}S]cysteine. 500-μl aliquots were dispensed in 1.88-cm^2 culture wells and treated either with GM-CSF or dexamethasone as indicated in the relevant figure legends. Cells were harvested and pelleted and FLAP protein was detected by immunoprecipitation essentially as previously described (6).

TCA Precipitation of Proteins. All steps were performed on ice. A small aliquot of each sample from metabolic labeling studies (2–5 μg of proteins) was pipetted into 1.5-ml (Eppendorf Inc., Fremont, CA), along with 100 μl H_2O, 100 μl of BSA as a carrier protein, and incorporation of ^{35}S into total neutrophil proteins was determined as previously described (14).

Two-dimensional Gel Electrophoresis and Western Blot Procedures. The cells were treated with rhGM-CSF as indicated, and processed for two-dimensional gel electrophoresis according to the method of O'Farrell (15). Equal amounts of total protein, as determined using a protein assay (Bio-Rad Laboratories, Richmond, CA) were loaded onto each gel. The first dimension was run under nonequilibrium conditions using 2% ampholite (1:1 [vol/vol] isoelectric ranges 4–8, and 8–10.5). The samples were migrated for 4,000 V-h. The second dimension was run on 15% gels, according to the method of Thomas and Kornberg (16). The proteins were transferred overnight at a 300 mA current setting onto an Immobilon-P (Millipore Corp.) blotting membrane. The transfer efficiency was visualized by Ponceau Red staining. The membranes were soaked for 1 h at 37°C in tris buffered saline (TBS) (25 mM Tris-HCl, pH 7.6, 0.2 M NaCl, 0.15% Tween 20) containing 2% gelatin (wt/vol) as a nonspecific site-blocking agent, and subsequently exposed to a 1:5,000 dilution of anti-FLAP polyclonal antibody for 1 h at 37°C. The membranes were then washed twice with 150 ml of TBS for 15 min at 25°C, and treated with a horseradish peroxidase–linked donkey anti–rabbit antibody (dilution of 1:7,500) in TBS containing 2% gelatin for 45 min, at 37°C. After two washes, the signal was revealed using the ECL kit, according to the manufacturer's instructions.

Isolation of Neutrophil Nuclei. Preparations of neutrophil nuclei were obtained as described by Woods et al. (17), with minor modifications. Briefly, neutrophil suspensions were centrifuged at 12,000 *g* for 5 s, supernatants were removed and cell pellets (10×10^6 cells) were vortexed (5 s), before the progressive addition of 300 μl of NP-lysis buffer (0.1% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA), supplemented with the following antiprotease cocktail: leupeptin (10 μg/ml); aprotinin (10 μg/ml); and phenylmethanesulphonyl fluoride (1 mM). The samples were kept 5 min on ice, then centrifuged at 500 *g* for 10 min, at 25°C. The supernatants (nonnuclear fraction), and

the pellets (nucleus-containing fraction), were immediately prepared for one-dimension SDS-PAGE (18) and Western blot, as described above. Each well was loaded with material corresponding to 5 × 10^5 cells.

Results

Effect of Various Cytokines on the Steady-State Level of FLAP mRNA. Different cytokines known to activate myeloid cells were assessed for their ability to induce an increase in the steady-state level of FLAP mRNA. Neutrophils were treated for a period of 3 h at 37°C with each of the following cytokines: IL-3; IL-6; IFN-γ; TNF-α; or GM-CSF (Fig. 1). This time point was chosen from the results of previous kinetic studies performed using lipopolysaccharide or GM-CSF as an activator (data not shown). Total RNA was then purified and the level of FLAP mRNA was determined by Northern blotting, using a 0.84-kb FLAP cDNA probe. Of all the cytokines tested, only GM-CSF, and to a lesser extent TNF-α, consistently induced a significant accumulation of FLAP mRNA. We therefore designed additional experiments in order to further characterize this effect of GM-CSF.

Neutrophils were treated for the indicated periods of time with 3 nM GM-CSF or its diluent (0.01% BSA) at 37°C. This concentration of the cytokine was chosen from the results of previous experiments on neutrophils performed in this laboratory using GM-CSF (11, 12). In these experiments, GM-CSF induced an increase in the steady-state level of FLAP mRNA in a time-dependent manner (Fig. 2). Over six independent experiments, a significant increase in FLAP mRNA was detectable between 30 min and 1 h of GM-CSF treatment. This stimulatory effect was observed for up to 4 h. After 16 h, the levels of FLAP mRNA were significantly lower. The stimulatory effect of GM-CSF was also dose dependent, with a maximal effect being observed using 3 nM of the cytokine (data not shown). Neutrophils were also preincubated with 20 μg/ml of the protein synthesis inhibitor, CX before being exposed to GM-CSF. CX failed to prevent the increased accumulation of FLAP mRNA induced by GM-CSF (Fig. 3). In fact, prior treatment of the cells with CX increased the accumulation of FLAP mRNA induced by GM-CSF in an additive manner. By itself, CX slightly increased the level of FLAP mRNA. Incubation of cells with 5 μg/ml of the transcription inhibitor, AD completely inhibited the accumulation of FLAP mRNA induced by GM-CSF.

Effect of GM-CSF on the Accumulation of Newly Synthesized FLAP. Neutrophils were treated with 3 nM GM-CSF for

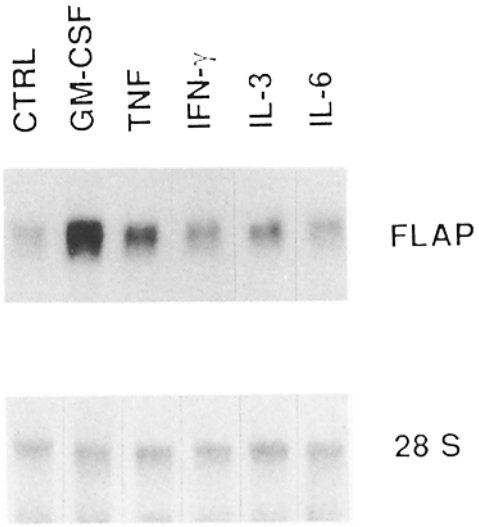

Figure 1. Effect of different cytokines on the steady-state level of FLAP mRNA. Neutrophils were incubated with the indicated cytokines for 3 h and total RNA was isolated. FLAP mRNA was detected by Northern blot. Integrity of the RNA and equal loading were verified by ethidium bromide staining, and hybridization with 28S ribosomal RNA probe, respectively. The cells were incubated with the following cytokines: GM-CSF (3 nM); TNF-α (100 ng/ml); IFN-γ (100 ng/ml); IL-3 (200 U/ml); or IL-6 (15 ng/ml). These data are from one representative experiment of four separate experiments.

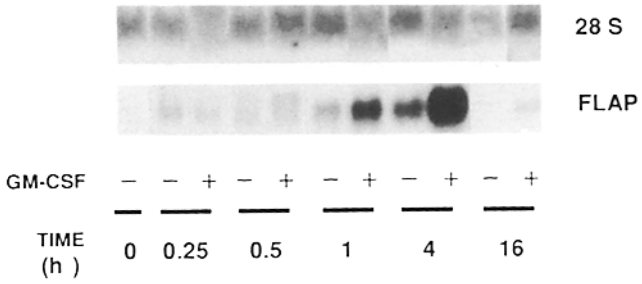

Figure 2. Effect of time of incubation with GM-CSF on the steady-state level of FLAP mRNA. Neutrophils were incubated for the indicated periods of time in the presence of 3 nM GM-CSF or its diluent, total RNA was isolated, and Northern blots performed to detect FLAP mRNA, as described in the legend to Fig. 1. These results are from one representative experiment of four separate experiments.

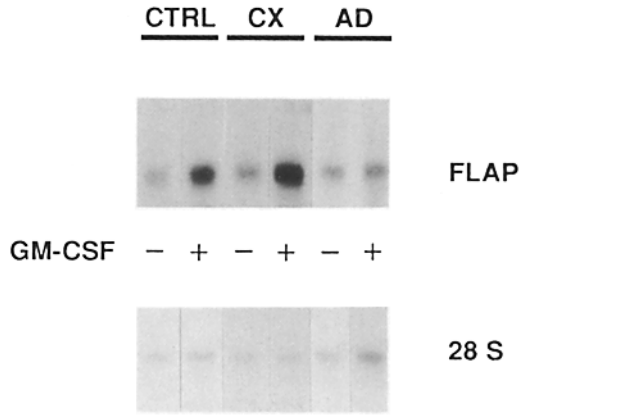

Figure 3. Effect of CX and AD on the steady-state level of FLAP mRNA. Neutrophils were incubated for 4 h with 3 nM GM-CSF in the presence or absence of either 20 μg/ml CX or 5 μg/ml AD. Total RNA was isolated, and Northern blots performed to detect FLAP mRNA, as described in the legend to Fig. 1. These results are from one representative experiment of seven separate experiments.

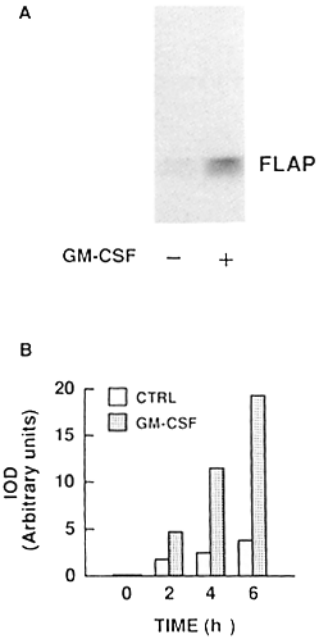

Figure 4. Kinetics of the effect of GM-CSF on the de novo synthesis of FLAP. Neutrophil suspensions in RPMI 1640 supplemented with 0.1% FCS and ^{35}S-labeled amino acids were treated with 3 nM GM-CSF (or its diluent) for various periods of time. Cells were then harvested and ^{35}S-labeled FLAP was immunoprecipitated, processed for SDS-PAGE, and analyzed by autoradiography as described in Materials and Methods. (*A*) Immunoprecipitation showing the de novo synthesis of FLAP by neutrophils in response to stimulation by GM-CSF for 6 h. (*B*) Densitometric analysis showing the effect of time of incubation with GM-CSF on the de novo synthesis of FLAP. These results are from one experiment, representative of five experiments. IOD, integrated optical density.

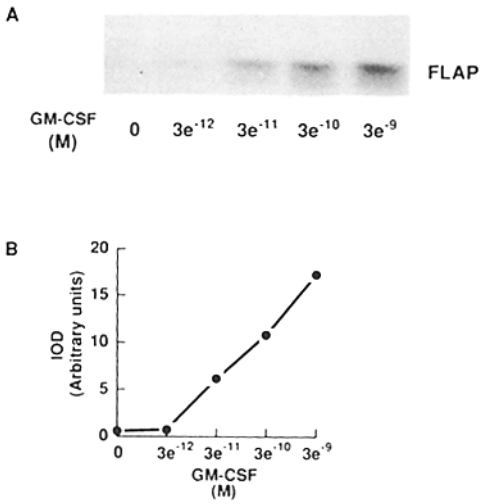

Figure 5. Effect of concentration of GM-CSF on the de novo synthesis of FLAP. Neutrophils were treated for 6 h in the presence of various concentrations of GM-CSF (or its diluent), and ^{35}S-labeled FLAP was immunoprecipitated as described in the legend to Fig. 4. (*A*) Immunoprecipition showing the de novo synthesis of FLAP by neutrophils in response to stimulation with increasing concentrations of GM-CSF. (*B*) Densitometric analysis of the autoradiograms shown in *A*. These results are from one experiment, representative of five separate experiments.

up to 6 h at 37°C, in the presence of [^{35}S]methionine and [^{35}S]cysteine, and FLAP immunoprecipitations were performed. GM-CSF enhanced the accumulation of newly synthesized FLAP (Fig. 4 *A*). An increased incorporation of ^{35}S amino acids into FLAP was consistently observed after 2 h of GM-CSF treatment, and continued to increase for up to 6 h in the presence of the cytokine (Fig. 4 *B*). GM-CSF consistently failed to induce a detectable accumulation of newly synthesized FLAP at time points shorter than 2 h (data not shown). Incubation of the cells with either AD or CX before treatment with GM-CSF completely blocked the increase in newly synthesized FLAP (data not shown).

Dose-response experiments were conducted in which cells were treated for 6 h in the presence of various concentrations of GM-CSF. The results of these experiments showed that the increased de novo synthesis of FLAP was dependent upon the concentration of GM-CSF. In three independent experiments, the minimum concentration required to induce the de novo synthesis of FLAP was 30 pM, and de novo synthesis was nearly maximal at 300 pM GM-CSF (Fig. 5, *A* and *B*).

To verify that the effect of GM-CSF on the de novo synthesis of FLAP was specific, total cell protein was obtained by TCA precipitation in each experiment, and the incorporation of [^{35}S]methionine and cysteine was determined. Over a total of 11 separate experiments in which neutrophils were exposed to GM-CSF for up to 6 h, no significant enhancement of the incorporation of [^{35}S]methionine and [^{35}S]cysteine into total neutrophil protein was observed. The value for GM-CSF–treated neutrophils was 104 ± 10% (mean ± SEM) of the control value.

Effect of GM-CSF on the Total FLAP Protein Content of Neutrophils. The effect of GM-CSF on the total cellular level of FLAP in neutrophils was investigated in control and GM-CSF–treated neutrophils by Western blot in order to verify

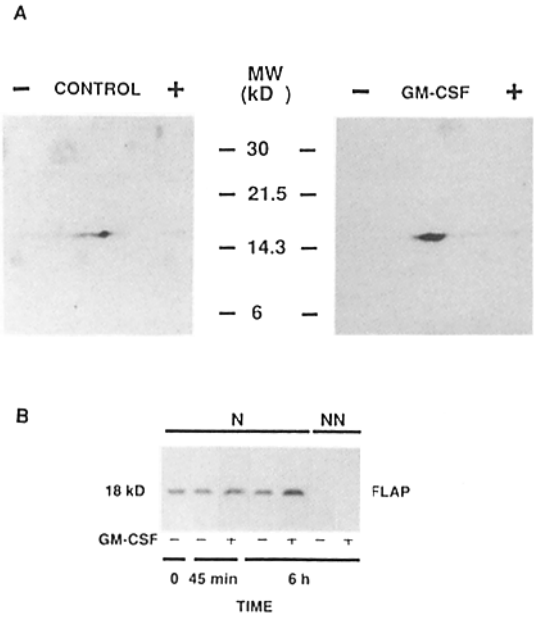

Figure 6. Effect of GM-CSF on the total cellular FLAP levels in whole neutrophils and neutrophil nuclei. (*A*) Neutrophils were incubated for 6 h in the absence (*left*), or presence of 3 nM GM-CSF (*right*). Equal amounts of total cellular proteins were then separated by two-dimensional gel electrophoresis and FLAP was detected by Western blot. This experiment is representative of five separate experiments. (*B*) Neutrophils were incubated with GM-CSF for indicated times, and nuclei were prepared and processed for SDS-PAGE as described in Materials and Methods. N, nuclear-containing fraction; NN, nonnuclear fraction. This experiment is representative of four separate experiments.

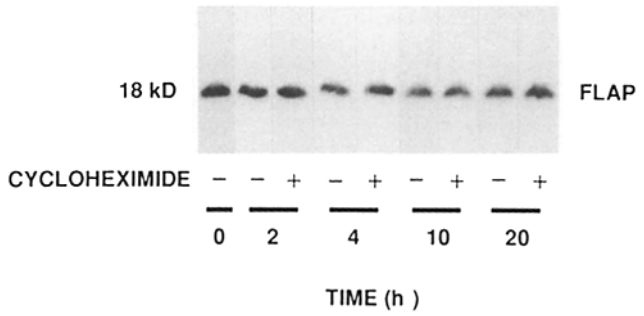

Figure 7. Assessment of FLAP stability. Neutrophils were incubated with (and without) 20 μg/ml CX for up to 20 h. Nuclei were then prepared and processed for SDS-PAGE as described in Materials and Methods. These results are from one representative experiment of four separate experiments.

whether the increased FLAP expression induced by GM-CSF was of sufficient magnitude to influence the total cellular pool of FLAP. Cells were therefore incubated for 6 h in the presence of GM-CSF or its diluent, and two-dimensional SDS-PAGE were performed. Equal amounts of total cellular proteins were loaded on each gel, as measured by a protein assay. Under these conditions, a single spot with a molecular mass of ~18 kD and an isoelectric point in the 7–9 range, corresponding to the migration point of FLAP, was detected (Fig. 6 *A*). Incubation with GM-CSF significantly increased the overall quantity of FLAP present in intact neutrophils (Fig. 6 *A*, *right*), relative to control cells. Densitometric analysis for FLAP revealed a 5.8-fold ± 2.5 (mean ± SEM, n = 5) increase in GM-CSF–treated neutrophils, as compared with the control.

In recent electron microscopy studies, FLAP was found to be localized in the nuclear envelope of neutrophils (17). We therefore performed additional experiments to assess that the increased expression of FLAP could be specifically observed in a neutrophil subcellular fraction containing intact nuclei. Neutrophils were treated with GM-CSF (or diluent), fractions containing nuclei as well as the corresponding nonnuclear fractions were obtained and processed for Western blot. In n = 4 experiments, FLAP expression was significantly increased in the nuclei of neutrophils treated with GM-CSF for 6 h, as compared with control neutrophils (Fig. 6 *B*). At no time was FLAP observed in the nonnuclear fraction.

Assessment of FLAP Stability in Neutrophils. Neutrophils were incubated in the presence (or absence) of 20 μg/ml CX for increasing periods of time (up to 20 h), and nuclear preparations were processed for Western blot. In four independent experiments, no significant decrease of FLAP levels could be observed, either in control or CX-treated cells (Fig. 7). This demonstrated that FLAP is a relatively stable protein in human neutrophils. It was concluded that the effect of GM-CSF on FLAP levels observed in short term exposure (6 h) of neutrophils to the cytokine were the consequence of enhanced de novo synthesis of the protein rather than protein stabilization.

Regulation of FLAP Expression by Dexamethasone. It has recently been shown that the FLAP gene contains a putative

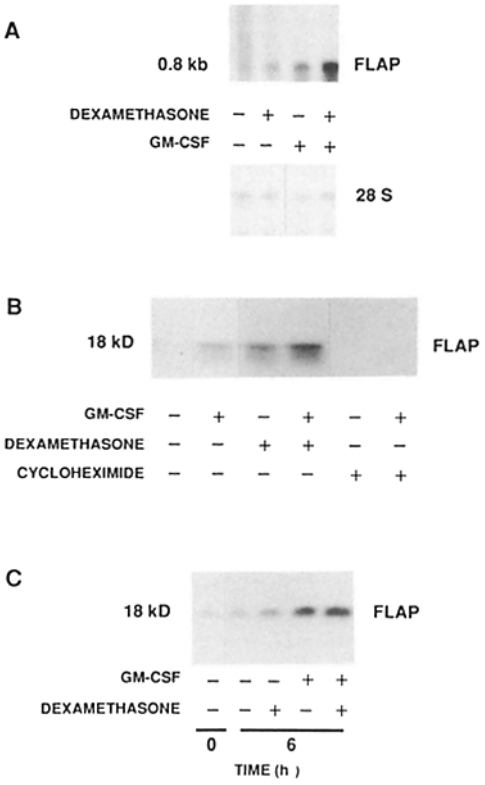

Figure 8. Effects of dexamethasone on FLAP expression. (*A*) Effect of dexamethasone on FLAP mRNA steady-state levels. Neutrophils were treated with 100 nM dexamethasone and/or 3 nM GM-CSF, for 4 h. Northern blots were then performed to determine the levels of FLAP mRNA. This result is from one experiment, representative of five separate experiments. (*B*) Effect of dexamethasone on the de novo synthesis of FLAP. Neutrophils were treated with 100 nM dexamethasone and/or 3 nM GM-CSF in the presence of [^{35}S]methionine and [^{35}S]cysteine, and FLAP was immunoprecipitated and analyzed as described in the legend to Fig. 4. Alternatively, neutrophils were pretreated with CX (20 μg/ml) before incubation with dexamethasone. These results are from one experiment, representative of four separate experiments. (*C*) Effect of dexamethasone on FLAP expression in neutrophil nuclei. Neutrophils were treated with GM-CSF and/or dexamethasone for 6 h and nuclei were prepared and processed as described in Materials and Methods. These results are from one experiment, representative of four separate experiments.

glucocorticoid-responsive element (GRE) in its 5′-regulatory region (10). In view of the fact that glucocorticoids are potent antiinflammatory agents and leukotrienes are considered proinflammatory, we investigated whether the synthetic glucocorticoid, dexamethasone, could downmodulate the expression of FLAP. Cells were therefore treated with 100 nM dexamethasone, in the presence or absence of 3 nM GM-CSF, for 4 h at 37°C. Under these conditions, dexamethasone by itself increased FLAP mRNA steady-state levels when compared with control samples, as determined by Northern blot (Fig. 8 *A*). Coincubation of GM-CSF and dexamethasone caused an additive increase in the level of the FLAP mRNA, when compared with the effect of either agent alone. Preincubation of the cells with the protein synthesis inhibitor, CX, did not block the effect of dexamethasone on the level of FLAP mRNA. In contrast, AD (5 μg/ml) completely blocked the

stimulatory effect of dexamethasone on the accumulation of FLAP mRNA (data not shown).

In protein-labeling experiments, in which both control- and GM-CSF–treated neutrophils were incubated for 4 h in the presence of dexamethasone, an increase in the de novo synthesis of FLAP was observed in dexamethasone-treated cells (Fig. 8 *B*). In these experiments, preincubation of the neutrophils with CX (20 μg/ml) completely abolished the de novo synthesis of FLAP. In addition, nuclear preparations from neutrophils incubated with 100 nM dexamethasone for 6 h showed a significant increase of FLAP level (Fig. 8 *C*), as compared with diluent-treated neutrophils.

Discussion

The leukotrienes are a family of lipid mediators involved in inflammation, allergy and asthma (1, 2). The synthesis of these compounds is critically dependent upon the activation of the arachidonate 5-LO. A growing body of evidence suggests that the participation of a recently discovered membrane-bound protein, termed FLAP, is required for leukotriene synthesis to occur in intact cells (6–9). Although the precise role of FLAP has not yet been defined, the latest evidence indicates that it may facilitate substrate presentation to the 5-LO (19). To date, most studies on FLAP have focused on the functional aspects of this molecule. Since little is currently known concerning the regulation of FLAP in terminally differentiated phagocytes, we have conducted studies to determine whether FLAP expression could be physiologically modulated in peripheral blood neutrophils, a cell type that is one of the major producers of LTB_4 in the immune system (2, 3).

In previous studies, we have shown that amidst a wide array of neutrophil agonists, GM-CSF was one of only three that were capable of stimulating a significant RNA synthesis in these cells, the other two being the formylated oligopeptide fMet-Leu-Phe, and the multifunctional cytokine TNF-α (11). However, we observed that GM-CSF was by far the most potent on the de novo synthesis of a select number of proteins (12). We and others subsequently identified several of these proteins as the IL-1 receptor antagonist (12), cationic antimicrobial protein (20), actin (21), and MHC class II (22). In the present report, we have determined that FLAP expression in neutrophils is also selectively upregulated by GM-CSF. Several other cytokines, including IFN-γ, IL-3, and IL-6, consistently failed to stimulate the accumulation of FLAP mRNA. This selective upregulation by GM-CSF of protein synthesis in neutrophils was further assessed in the present study when we examined the overall incorporation of [^{35}S]methionine and [^{35}S]cysteine into total cellular proteins. The amount of [^{35}S]amino acids incorporated into the TCA-precipitable fraction in GM-CSF–treated neutrophils was not significantly different from that in diluent-treated cells, yet in the same experiments, a significant de novo synthesis of FLAP was observed in GM-CSF–treated cells. This latter result is important in that it confirms that the effect of GM-CSF on protein expression in neutrophils is relatively specific and restricted to a limited number of target proteins (21, 23). Whereas the mechanism by which GM-CSF increases the steady-state level of FLAP mRNA has not been conclusively established, pretreatment of human neutrophils with CX failed to prevent the upregulation of FLAP mRNA by GM-CSF, suggesting that the effect of GM-CSF on FLAP mRNA is not dependent on the synthesis of other proteins. Moreover, the increased expression of FLAP mRNA was completely inhibited by the transcription inhibitor AD, suggesting increased FLAP mRNA synthesis. Further studies are required to define the exact mechanism by which GM-CSF enhances FLAP expression in neutrophils.

In view of the fact that FLAP is crucial for leukotriene synthesis to occur in intact neutrophils, the question of the potential physiological relevance of an increased de novo synthesis of FLAP in neutrophils in response to GM-CSF arises. Over the last few years, several reports have documented that pretreating human neutrophils with GM-CSF strongly enhances leukotriene synthesis in response to second agonists. In these studies, a maximal priming for leukotriene synthesis was observed when neutrophils were pretreated with GM-CSF for between 30 min and 1 h (depending on the conditions of incubation), after which the priming effect rapidly decreased (24–26). In the present study, an increase in the de novo synthesis of FLAP was not observed until after 2 h of incubation with GM-CSF. Moreover, the level of FLAP protein continued to increase for the total length of the experiment (6 h) after exposure to GM-CSF. The difference between the time course reported for the priming by GM-CSF for increased leukotriene synthesis, and that for the de novo synthesis of FLAP, indicates that the effect of GM-CSF on FLAP expression is unlikely to be involved in the priming for increased leukotriene synthesis observed in short-term incubations in vitro. However, it must be stressed that little is known concerning the turnover and fate of proteins of the 5-LO pathway, including FLAP, over prolonged periods of time in neutrophils, nor are the long-term effects of GM-CSF on leukotriene synthesis by neutrophils in vivo known. Since leukotriene synthesis requires the participation of FLAP, our data raise the possibility that the increase in FLAP expression in response to GM-CSF could play a significant long-term role in enhancing or maintaining leukotriene synthesis by neutrophils, in response to subsequent stimulation. The ability of GM-CSF to increase neutrophil survival in vitro (27), possibly via inhibition of apoptosis (28), further supports the contention that prolonged synthesis of FLAP may occur in vivo. Such a scenario may be particularly relevant at sites of acute and chronic inflammation, where neutrophils may be subject to long-term exposure to relatively high levels of GM-CSF (29).

Glucocorticoids are a class of widely used antiinflammatory drugs that profoundly affect the inflammatory response by exerting both inhibitory and stimulatory effect on the expression of certain genes (30). Kennedy et al. (10), have recently cloned, sequenced, and begun to functionally characterize the 5′-regulatory region of the FLAP gene. Several potential *cis*-regulatory regions were identified, including potential binding sites for the nuclear transcription factor AP-2 at positions −482 to −475 bp, and for the glucocorticoid

receptor complex at positions −563 to −556 bp (10). The presence of this latter *cis*-regulatory element prompted us to examine the effect of the synthetic glucocorticoid dexamethasone on FLAP expression in neutrophils. In these studies, we sought to determine whether dexamethasone would inhibit FLAP expression induced by GM-CSF, an effect which would have been in keeping with an antiinflammatory action of dexamethasone. However, we observed that incubation of neutrophils with dexamethasone resulted in a direct increase in the FLAP mRNA level and in the expression of FLAP. Moreover, the combined effect of dexamethasone and GM-CSF was to additively enhance FLAP expression. The results of these experiments reemphasize the complexity of the action of glucocorticoids by demonstrating that not all of the effects of these compounds are necessarily antiinflammatory in nature.

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Address correspondence to Dr. Shaun R. McColl, The John Curtin School of Medical Research, The Australian National University, Acton, Australian Capital Territory, Australia, 2601.

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