Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9190–9194, September 1994
Cell Biology

Mapping of the C5a receptor signal transduction network in human neutrophils

(Ras/Raf-1/B-Raf/mitogen-activated protein kinase)

ANNE METTE BUHL*†, NATALIE AVDI‡, G. SCOTT WORTHEN‡§, AND GARY L. JOHNSON†¶

*Division of Biostructural Chemistry, Department of Chemistry, University of Aarhus, DK-8000 Aarhus, Denmark; †Division of Basic Sciences and ‡Department of Medicine, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson Street, Denver, CO 80206; and Departments of ¶Pharmacology and §Medicine, University of Colorado Medical School, Denver, CO 80262

Communicated by Philippa Marrack, June 6, 1994

ABSTRACT **Human neutrophils respond to chemoattractants, resulting in their accumulation at an inflammatory site. Chemoattractants such as the C5a peptide, derived from the C5 complement factor, bind to inhibitory guanine nucleotide binding protein (G_i)-coupled seven membrane-spanning receptors expressed in neutrophils. C5a receptor activation results in the G_i-dependent activation of the mitogen-activated protein (MAP) kinase pathway in human neutrophils. C5a receptor ligation activates both B-Raf and Raf-1, with B-Raf activation overlapping but temporally distinct from that of Raf-1. B-Raf and Raf-1 both efficiently phosphorylate MAP kinase kinase (MEK-1). C5a also stimulates guanine nucleotide exchange and activation of Ras. Ras and Raf activation in response to C5a involves protein kinase C-dependent and -independent pathways. Activation of both Raf-1 and B-Raf was inhibited by protein kinase A stimulation, consistent with the inhibitory effects of increased cAMP levels on neutrophil function. The findings define a functional signal transduction pathway linking the neutrophil C5a chemoattractant receptor to the regulation of Ras, B-Raf, Raf-1, and MAP kinase.**

Neutrophils are critical for the nonspecific host defense against infection (1). Chemoattractants released at the inflammatory site recruit neutrophils by binding to specific receptors expressed on the neutrophil surface. Chemoattractant receptors for C5a and fMet-Leu-Phe are seven membrane-spanning receptors coupled to inhibitory heterotrimeric guanine nucleotide binding proteins (G_i proteins) (2, 3). Little is known about the neutrophil signal transduction pathways regulated by G_i-coupled chemoattractant receptors, except for the stimulation of phospholipase Cβ (PLCβ) and phosphatidylinositol 3-kinase activities (4–6) followed by activation of phospholipase A_2 (PLA_2) and phospholipase D (7). Other signal transduction events responsible for chemoattractant regulation of neutrophil function remain obscure.

Recent findings in fibroblasts have demonstrated that G_i-coupled seven membrane-spanning receptors regulate the mitogen-activated protein (MAP) kinase pathway (8). MAP kinase is known to phosphorylate and regulate a number of proteins potentially important in chemoattractant regulation of neutrophils. Defined MAP kinase substrates include the microtubule-associated proteins MAP-2 (9) and tau (10, 11); cytoplasmic PLA_2, which is known to be activated in response to chemoattractants (12, 13); other serine-threonine kinases such as Rsk 90 (14); and several transcription factors including c-Myc, c-Fos, c-Jun, Elk-1, ATF2, and NF-IL6 (15).

In this report, we define a MAP kinase signal transduction pathway activated by C5a receptor ligation that requires a functional G_i protein. The results define a sequential protein phosphorylation response pathway involved in chemoattractant stimulation of mature, differentiated neutrophils.

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MATERIALS AND METHODS

Preparation of Human Neutrophils. Human neutrophils were prepared by a method that minimizes lipopolysaccharide exposure, using plasma/Percoll gradients as described by Haslett *et al.* (16).

To evaluate the ADP-ribosylation levels obtained with pertussis toxin (PTx) treatment, membranes (20 μg) (17) from PTx-treated or untreated cells were incubated for 1 hr with dithiothreitol-preactivated PTx in the presence of 10 μCi of [^{32}P]NAD^+ (1 Ci = 37 GBq). Reactions were stopped by trichloroacetic acid precipitation of the membranes; the protein was resuspended in SDS sample buffer and fractionated by SDS/PAGE. ADP-ribosylated α_{i2} for each condition was quantitated by phosphorimaging.

Measurement of Ras Activation. Activation of Ras was determined by measurement of agonist-dependent GTP loading onto Ras basically as described (2, 18). PEI-cellulose thin-layer chromatography in 0.75 M KH_2PO_4 (pH 3.4) was used to resolve guanine nucleotides, and Ras-bound [α^{32}P]GDP and [α^{32}P]GTP was determined by phosphorimaging.

Measurement of Raf-1 and B-Raf Activity. For measurement of Raf-1 and B-raf activity, 4×10^7 cells were used per sample and the assay was performed as described (2, 19).

Tryptic Phosphopeptide Mapping. MEK-1 was used as substrate in an *in vitro* kinase assay for Raf-1 or B-Raf. Phosphorylated MEK-1 was resolved by SDS/PAGE, transferred to nitrocellulose, and identified by autoradiography. The MEK-1 bands were excised and digested by the method of Luo *et al.* (20). The tryptic phosphopeptides were analyzed by electrophoresis (20 min, 2.0 kV at pH 1.9) followed by chromatography in isobutyric acid/pyridine/acetic acid/1-butanol/H_2O, 65:5:3:2:29 (21).

MAP Kinase Assay. MAP kinase activity was measured with 8×10^7 cells per sample and the assay was performed as described by Gupta *et al.* (22).

All experiments involving the measurement of Ras, Raf-1, B-Raf, and MAP kinase were performed at least three times with neutrophils from different donors with similar results.

RESULTS

MAP Kinase Activation in Response to C5a. C5a ligation of its receptor stimulates MAP kinase activity in primary human

Abbreviations: G_i protein, inhibitory guanine nucleotide binding protein; PLCβ, phospholipase Cβ; PLA_2, phospholipase A_2; MAP kinase, mitogen-activated protein kinase; PTx, pertussis toxin; PMA, phorbol 12-myristate 13-acetate; CP-cAMP, 8-[(4-chlorophenyl)thio]adenosine 3′,5′-phosphate.

neutrophils (Fig. 1*A*). Similar to the chemoattractant activation of PLCβ (4) and phosphatidylinositol 3-kinase (5, 6), the C5a activation of MAP kinase was sensitive to pretreatment of neutrophils with PTx (Fig. 1*B*). The PTx inhibition of MAP kinase activation is consistent with the C5a receptor being dominantly coupled to G_i proteins in the human neutrophil (23). MAP kinase stimulation in response to C5a is rapid and transient; maximal MAP kinase activity is observed within 3 min and returns to near basal levels by 10–30 min even in the continued presence of maximally effective C5a.

B-Raf and Raf-1 Are Activated in Response to C5a. Raf-1 has been defined as an upstream regulator of MEK-1 in several fibroblast cell lines (24, 25). Raf-1 is a serine-threonine protein kinase shown to interact with Ras·GTP. C5a ligation of its receptor activated Raf-1 in human neutrophils (Fig. 2 *A* and *C*). The time course of Raf-1 activation was essentially the same as that observed for MAP kinase (compare Figs. 1*A* and 2*A*). B-Raf is a serine-threonine protein kinase homolog of Raf-1 (26, 27). Whereas Raf-1 has been shown to regulate MEK-1 and to couple to Ras·GTP, no regulatory role for B-Raf has been defined. Neutrophils express B-Raf, and C5a ligation of its receptor activates B-Raf kinase activity toward MEK-1 (Fig. 2 *B* and *C*).

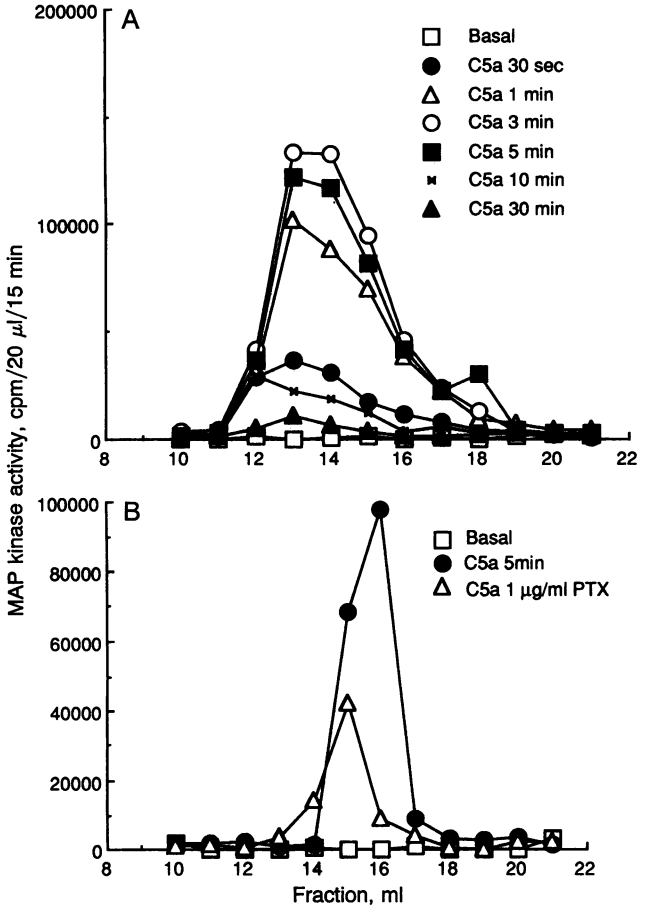

FIG. 1. (*A*) C5a stimulates MAP kinase activity in human neutrophils. Neutrophils that had been preincubated with protease inhibitors were stimulated with a final concentration of 50 nM C5a for various times and were then assayed for MAP kinase activity. Phorbol 12-myristate 13-acetate (PMA) stimulation (10 ng/ml) resulted in maximal MAP kinase activation at 3 min (data not shown). (*B*) Neutrophils were preincubated with PTx (1 μg/ml) for 2.5 hr at 37°C before being stimulated with C5a, lysed, and applied to the Mono Q column. MAP kinase activity was inhibited by 60% after PTx treatment relative to control cells in the experiment shown. The Mono Q columns used in *A* and *B* were different, accounting for the slightly shifted elution profiles of MAP kinase activity.

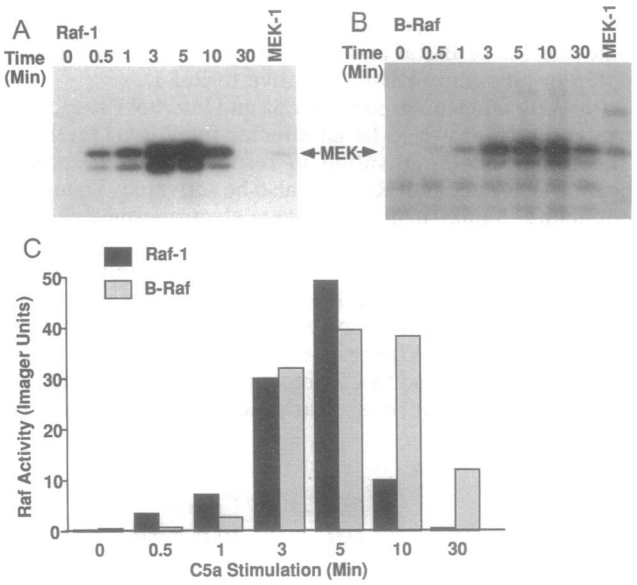

FIG. 2. C5a stimulates the activity of Raf-1 and B-Raf in human neutrophils. Neutrophils (4×10^7 per sample) were stimulated for the indicated time with C5a. Cell lysates were prepared and Raf-1 or B-Raf was selectively immunoprecipitated. Autoradiographs show the time-dependent phosphorylation of MEK-1 by Raf-1 (*A*) and B-Raf (*B*). Due to proteolytic activity in the neutrophil lysate, a doublet of cleaved and uncleaved recombinant kinase inactive MEK-1 is seen. Imager units represent the levels of MEK-1 phosphorylation visualized by phosphorimaging analysis (*C*). Typically, the Raf-1 immunoprecipitates had 5–7 times more MEK-1 kinase activity relative to B-Raf immunoprecipitates. This appears to be related in part to a greater abundance of Raf-1 relative to B-Raf and the properties of the antibodies. No cross-reactivity between Raf-1 and B-Raf was detectable with either antibody. Neither antibody immunoprecipitated MEK-1 or MAP kinase from neutrophil lysates.

Phosphopeptide mapping demonstrated that Raf-1 and B-Raf phosphorylated the same sites on MEK-1 (Fig. 3). The time course of B-Raf activation was different from that for both Raf-1 and MAP kinase. B-Raf activation is slower in onset but longer in duration than the activation of Raf-1 and MAP kinase. This finding was reproducible in several different experiments and suggests a differential control of B-Raf in the

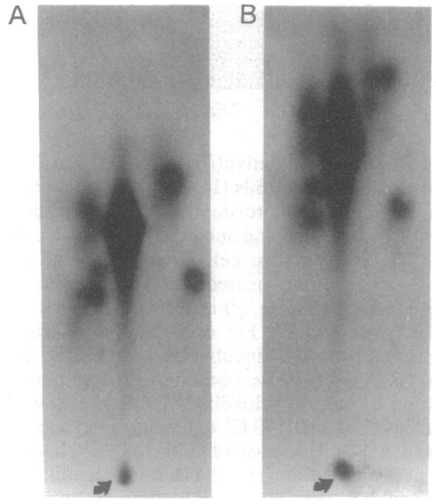

FIG. 3. Autoradiograms of two-dimensional tryptic phosphopeptide maps of kinase inactive MEK-1. (*A*) Kinase inactive MEK-1 phosphorylated by Raf-1. (*B*) Kinase inactive MEK-1 phosphorylated by B-Raf. Maps are identical, indicating that both Raf enzymes phosphorylate the same site(s) on MEK-1. Arrows indicate origin.

C5a regulation of neutrophil function relative to Raf-1. The time courses of B-Raf and MAP kinase activation suggest a different function for B-Raf relative to Raf-1.

Ras Activation in Response to C5a and Phorbol Esters. Raf-1 was recently shown to be an effector for Ras·GTP (28–32). The robust Raf-1 activation in response to C5a receptor ligation suggested that Ras may also be activated. To test this possibility, human neutrophils were electropermeabilized to introduce [α-^{32}P]GTP. The exchange of [α-^{32}P]GDP for [α-^{32}P]GTP in response to C5a was then monitored. The electropermeation protocol was necessary because of the difficulty in labeling the nucleotide pool with $^{32}P_i$, as primary human neutrophils lose responsiveness within 4–6 hr after isolation. In response to C5a receptor ligation, Ras guanine nucleotide exchange was clearly activated (Fig. 4*A*). The

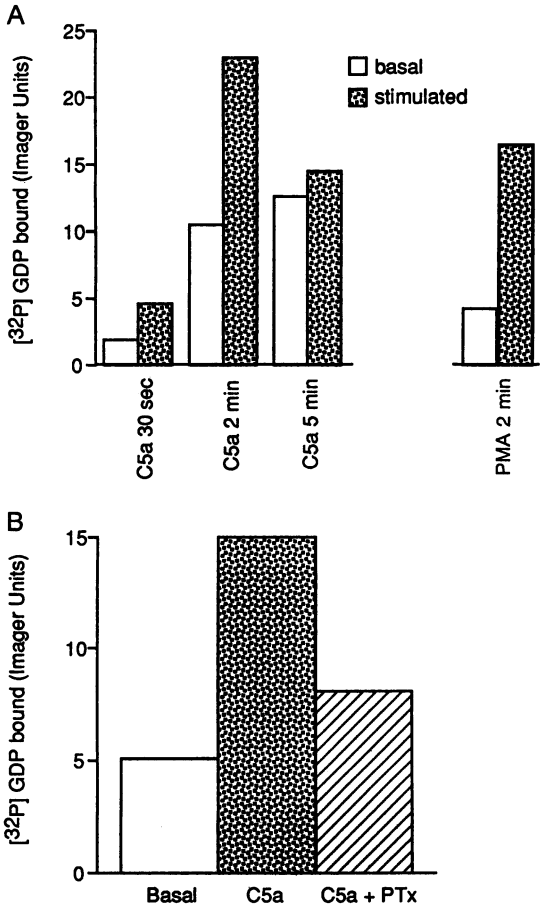

FIG. 4. C5a-dependent activation of Ras GDP/GTP exchange in human neutrophils. Neutrophils (10^7 per sample) were preincubated with Krebs–Ringer/phosphate/dextrose (KRPD) and protease inhibitors for 0.5 hr before being subjected to two electric discharges of 1.75 kV. The resuspended cells were incubated with 5 μCi of [α-^{32}P]GTP on ice for 1 min, heated to 37°C for 15 sec, and incubated with C5a (final concentration, 50 nM) or PMA (final concentration, 10 ng/ml) for various times. For each time point, basal GDP/GTP exchange was determined by incubation with KRPD. Quantitation of radiolabeled GDP and GTP was accomplished by phosphorimaging. (*A*) Stimulation of Ras·GTP loading by C5a and the phorbol ester PMA. Basal level of GDP/GTP exchange increased linearly with C5a. At 30 sec, a 2-fold increase of GDP/GTP exchange is seen; this is sustained at 2 min, but at 5 min the Ras·GTP loading is inactivated. PMA (10 ng/ml) activates Ras·GTP loading at 2 min of stimulation (this is a different experiment than the results shown for C5a, which accounts for the difference in basal Ras·GTP loading at 2 min for PMA relative to C5a stimulation). (*B*) Neutrophils were treated with PTx (1 μg/ml) in KRPD plus protease inhibitors for 2.5 hr. Ras activation in response to C5a was inhibited ≈70% relative to C5a stimulation of control cells.

activation of Ras in response to C5a was decreased ≈70% by prior PTx treatment of the neutrophils (Fig. 4*B*), consistent with the C5a activation of Ras requiring the coupling of PTx-sensitive G_i proteins to the C5a receptor.

In T and B lymphocytes phorbol ester stimulation of protein kinase C leads to Ras activation (33, 34). The phorbol ester PMA also stimulates Ras activation in human neutrophils (Fig. 4*A*). The ability of PMA to activate Ras in human neutrophils indicated that stimulation of PLCβ in response to C5a could contribute to this response. C5a receptor stimulation of G_i generates α_i·GTP and $\beta\gamma$ subunits. The cell-specific PLCβ type II that is activated by $\beta\gamma$ was cloned from HL-60 cells (35). Neutrophils express a PLCβ that is stimulated by G protein $\beta\gamma$ subunits (36), which would subsequently lead to the activation of protein kinase C in response to C5a.

Using Raf activation as a readout, the protein kinase C inhibitor GF109203X (37) inhibited the PMA response by >90% (Fig. 5*A*). The C5a receptor activation of Raf was inhibited only 40–50% by treatment of human neutrophils with GF109203X. This contrasts with the ability of PTx (1 μg/ml) treatment to inhibit ≈75% of the C5a receptor activation of both Raf-1 and B-Raf (Fig. 5*B*). These findings are consistent with protein kinase C-dependent and independent pathways leading to activation of the Ras/Raf/MAP kinase regulatory kinase cascade. To obtain maximal responsiveness of the kinase cascade, contributions from both pathways appear necessary.

Protein Kinase A Regulation of the Raf/MAP Kinase Pathway. Recently it has been shown that there is cross-talk between the MAP kinase signaling pathway and the adenylyl cyclase signaling pathway (38–40). In neutrophils, pretreatment with the cAMP analogue 8-[(4-chlorophenyl)thio]adenosine 3′,5′-phosphate (CP-cAMP), inhibits the activation of Raf-1 and B-Raf in response to C5a. As shown in Fig. 6, the time course of this inhibition is different for Raf-1 and B-Raf; Raf-1 inhibition is transient, being maximal after 3 min of incubation with CP-cAMP and returning toward control values after 15 min. In contrast, B-Raf activity remains inhibited during the time period of neutrophil incubation with CP-cAMP. This finding indicates that Raf-1 and B-Raf kinase activities are differentially regulated by protein kinase A. B-Raf does not have a consensus protein kinase A phosphorylation site near its N terminus. This contrasts with the proposed regulation of Raf-1 by phosphorylation at Ser-43 in its N-terminal regulatory domain (40). The mechanism for B-Raf regulation by protein kinase A is presently unclear.

DISCUSSION

Fig. 7 outlines the hypothesized pathway leading to MAP kinase activation in response to C5a. Both α_i·GTP and $\beta\gamma$ subunits are predicted to be involved in activating the Ras/Raf/MAP kinase pathway. Ras GTP loading stimulated in response to either α_i·GTP or $\beta\gamma$ subunits could involve Ras exchange factors or GAP (Ras GTPase activating protein) regulatory functions (41). Protein kinase A activation appeared to uncouple both Raf-1 and B-Raf activation in response to C5a; the uncoupling of Raf-1 was transient, while the uncoupling of B-Raf persisted for a longer time.

We and others recently published results showing the possible involvement of the $G\alpha_{16}$ polypeptide in C5a receptor signaling (42, 43). By coexpression of the C5a receptor and α_{16}, we could stimulate the activation of PLCβ in HEK293 cells. $G\alpha_{16}$ signaling, which could represent a PTx-insensitive pathway, does not seem to play a role in mature neutrophils; we were unable to detect expression of α_{16} in neutrophil membranes by immunoblotting (data not shown). During differentiation of precursor cells to neutrophils, the expression of the C5a receptor increases (3). The opposite is the

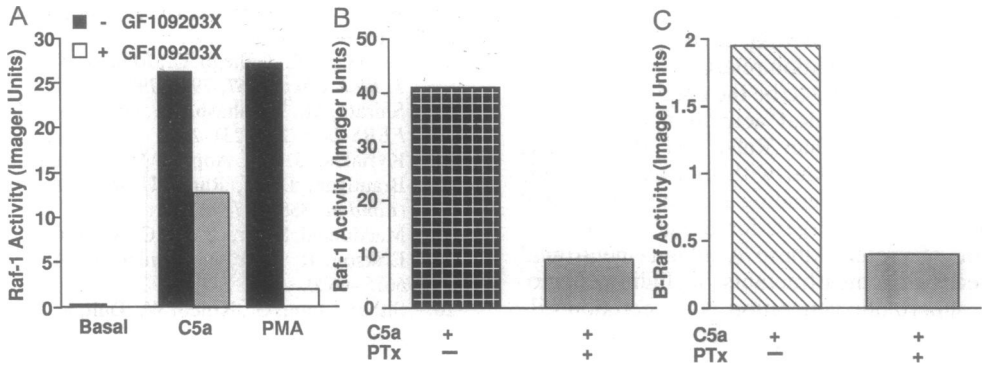

FIG. 5. (*A*) The protein kinase C inhibitor GF109203X inhibits PMA and blunts C5a stimulation of Raf-1. Neutrophils were incubated with Krebs–Ringer/phosphate/dextrose (KRPD), protease inhibitors, and the protein C inhibitor GF109203X (2 μM) for 0.5 hr before stimulation with C5a (5 min) and measurement of Raf-1 activity. (*B* and *C*) PTx pretreatment partially blocks activation of both Raf enzymes by C5a. Neutrophils were preincubated in KRPD with protease inhibitors and PTx (final concentration, 1 μg/ml). In the experiment shown, Raf-1 and B-Raf activation were inhibited by $\approx$75%.

case for the expression of α_{16} (44); compared to HL-60 cells α_{16} expression is undetectable in mature neutrophils. This argues that α_{16} does not play a significant role in signaling for the mature neutrophil.

The inhibition of C5a receptor-stimulated Ras/Raf and MAP kinase activity was not absolute with PTx treatment. To maintain neutrophil viability, it was not possible to incubate the cells with PTx for >2.5 hr. *In vitro* [^{32}P]ADP-ribosylation of α_i protein in membranes prepared from toxin-treated and control neutrophils was used to monitor the level of *in vivo* ADP-ribosylation. ADP-ribosylation of α_i was diminished in toxin-treated cells by 70–75% of the total α_i protein that was capable of being ADP-ribosylated in membranes from control cells. The 20–30% of non-ADP-ribosylated α_i protein probably accounts for the residual C5a receptor signaling observed in PTx-treated neutrophils. Ras·GTP loading and Raf-1 activation were inhibited to similar degrees (70–75%) by PTx treatment of neutrophils, consistent with G_i being required for regulation of both Ras and Raf.

Previously, it was shown that chemoattractants activated PLCβ and promoted the mobilization of intracellular Ca^{2+} in a G_i-dependent fashion (4, 23). Concurrent stimulation of PLA_2 and phospholipase D is involved in the numerous physiological responses seen upon stimulation of the neutrophil. The regulation of the Ras/Raf-1/MAP kinase pathway by chemoattractants in neutrophils identifies a major signal transduction system defined previously in other cell types to be involved in cytoskeletal assembly (45, 46) and the regulation of PLA_2 and arachidonic acid release (13). In fibroblasts a functional Ras protein is apparently not required for Rho and Rac activation in response to growth factors (47). Rho proteins are involved in the regulation of actin fiber assembly and the formation of focal adhesions (46). Rac proteins are believed to stimulate actin assembly associated with pinocytosis and membrane ruffling (47). Rac proteins are also important in regulation of the NADPH oxidase system in neutrophils (48). Recently, it was reported that arachidonic acid was a potent regulator of Rac·GDI (GDP dissociation inhibitor) complexes (49), suggesting that the regulation of PLA_2 is critical in neutrophil function. The cytoplasmic PLA_2 expressed in neutrophils is a substrate for p42 MAP kinase (12, 13). Phosphorylation of PLA_2 by MAP kinase increases its V_{max} in response to elevated intracellular calcium (12, 13), producing a greater arachidonic acid release. The G_i-dependent regulation of Ras and MAP kinase activities is therefore predicted to play a role in regulation of cytoskeletal changes and superoxide generation in neutrophils in response to chemoattractants.

The finding that B-Raf is activated in response to chemoattractants, in addition to Raf-1, indicates that the signal transduction network regulated by G_i-coupled C5a receptors involves several serine-threonine protein kinases. B-Raf is capable of phosphorylating MEK-1 at the same sites as Raf-1. We have previously demonstrated that phosphorylation of

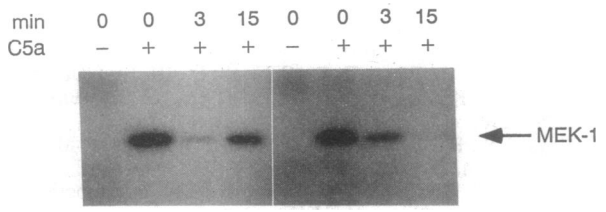

FIG. 6. Activation of protein kinase A reduces the activity of both Raf-1 (*Left*) and B-Raf (*Right*) in response to stimulation with C5a. Neutrophils preincubated for various times with CP-cAMP at a final concentration of 30 μM were stimulated with C5a (5 min), and Raf activation was subsequently determined. While the inhibition of Raf-1 is transient, the B-Raf activity remains inhibited after 15 min of incubation with CP-cAMP.

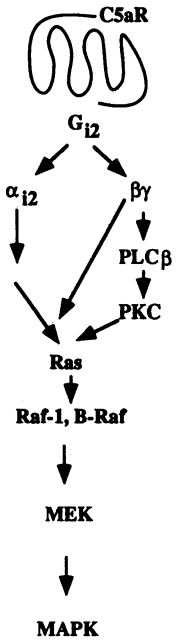

FIG. 7. Proposed model for C5a stimulation of the Ras/Raf/MAP kinase (MAPK) pathway in human neutrophils. G_{i2} is the predominant G protein responsible for the effects seen upon stimulation of the C5a receptor (C5aR). PKC, protein kinase C.

MEK-1 at these sites by Raf-1 and B-Raf increases MEK-1 phosphorylation and activation of MAP kinase (50). The time course of B-Raf and Raf-1 activation relative to MAP kinase suggests that Raf-1 is more likely to be the major regulator of MAP kinase in neutrophils. B-Raf activation is slower in onset and longer in duration than Raf-1 and MAP kinase activities, which closely correlate with one another. This suggests that other substrates including additional MEK and MAP kinase proteins may be targets for B-Raf regulation.

cAMP activation of protein kinase A inhibits neutrophil functions regulated by chemoattractants, including chemotaxis, adherence, superoxide generation, and secretion (51, 52). Previously, it was shown that elevated levels of cAMP inhibited fMet-Leu-Phe-mediated phospholipase D activation by an unknown mechanism (53). Although there are undoubtedly many substrates for protein kinase A in neutrophils, increased protein kinase A activity inhibits the stimulation of both Raf-1 and B-Raf in response to C5a. Thus, it is likely that the cAMP-mediated inhibition of Raf-1 and B-Raf are important in modulating the chemoattractant responses in neutrophils.

This work was supported by National Institutes of Health Grants GM30324, DK37871, CA58187, HL34303, and HL40478. A.M.B. is the recipient of a grant from the Danish Natural Science Research Council.

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