Mitogen-activated Protein Kinase in Neutrophils and Enucleate Neutrophil Cytoplasts

EVIDENCE FOR REGULATION OF CELL-CELL ADHESION

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We employed neutrophils and enucleate neutrophil cytoplasts to study the activation of the mitogen-activated protein kinases (MAPks) p44^{erk1} and p42^{erk2} in neutrophils by inflammatory agonists that engage G protein-linked receptors. Formyl-methionyl-leucyl-phenylalanine (FMLP) rapidly and transiently activated MAPK in neutrophils and cytoplasts, consistent with a role in signaling for neutrophil functions. FMLP stimulated p21^{ras} activation in neutrophils and Raf-1 translocation from cytosol to plasma membrane in cytoplasts, with kinetics consistent with events upstream of MAPK activation. Insulin, a protein tyrosine kinase receptor (PTKR) agonist, stimulated neutrophil MAPK activation, demonstrating an intact system of PTKR signaling in these post-mitotic cells. FMLP- and insulin-stimulated MAPK activation in cytoplasts were inhibited by B_{t}cAMP, consistent with signaling through Raf-1 and suggesting a mechanism for cAMP inhibition of neutrophil function. However, B_{t}cAMP had no effect on FMLP-stimulated MAPK activation in neutrophils. The extent of MAPK activation by various chemoattractants correlated with their capacity to stimulate neutrophil and cytoplast homotypic aggregation. Consistent with its effects on MAPK, B_{t}cAMP inhibited FMLP-stimulated aggregation in cytoplasts but not neutrophils. Insulin had no independent effect but primed neutrophils for aggregation in response to FMLP. Our studies support a p21^{ras}-Raf-1-dependent pathway for MAPK activation in neutrophils and suggest that neutrophil adhesion may be regulated, in part, by MAPK.

The mitogen-activated protein kinases (MAPks)\(^{1}\) p44^{erk1} and p42^{erk2} are serine/threonine kinases that participate in cell signaling for growth and differentiation (1). The most completely elucidated pathway for p44^{erk1} and p42^{erk2} activation utilizes protein tyrosine kinase receptors (PTKR) to activate MAPK through p21^{ras} and Raf-1. In this pathway, ligation of the PTKR results in interaction of the receptor with a complex of the adaptor protein Grb2 (2) and SOS, a guanine nucleotide exchange factor (3). These interactions bring SOS into proximity of p21^{ras} (4, 5), stimulating GTP/GDP exchange (6). Activated p21^{ras} recruits the serine/threonine kinase Raf-1 to the plasma membrane (PM) where it is activated (7–9). Activated Raf-1 phosphorylates a dual threonine/tyrosine kinase, MAPK or Erk kinase (10), which in turn phosphorylates and activates p44^{erk1} and p42^{erk2} (11). Raf-1 appears to play a pivotal role in this pathway because its activation can be negatively regulated by cAMP-dependent protein kinase A (PKA) (12, 13). Among other signaling pathways leading to MAPK activation are those activated by G protein-linked receptors, including receptors for lysophosphatidic acid (LPA) (14), acetylcholine (15), and thormbin (16). Whereas LPA and acetylcholine activate MAPK via p21^{ras} (14, 16), the thrombin receptor can activate MAPK through a p21^{ras}/Raf-1-independent pathway (17, 18).

Circulating neutrophils are terminally differentiated, post-mitotic phagocytes that constitute the first line of host defense against microorganisms. In contrast to dividing cells that respond slowly to mitogens, neutrophils respond rapidly to inflammatory stimuli. One class of neutrophil agonists, the chemoattractants, engage seven transmembrane-spanning domain receptors that activate G\(_{\text{pro}}\) proteins. The only well-documented effector downstream of neutrophil G\(_{\text{pro}}\) is phospholipase C\(_{\text{b}}\), a regulatory enzyme not directly linked to the MAPK pathway. Nevertheless, the chemoattractant N-formyl-methionyl-leucyl-phenylalanine (FMLP) has been shown to activate MAPK (19) and to stimulate MAPK autophosphorylation in neutrophils in a pertussis toxin-sensitive fashion (20). MAPKs thus represent candidate effectors for the signaling pathway(s) leading from G protein activation to rapid neutrophil responses. Because currently understood mechanisms of neutrophil activation fail to explain the observation that agents that elevate intracellular cAMP inhibit some chemoattractant-stimulated neutrophil responses, the possibility that chemoattractants activate MAPK's in a p21^{ras}/Raf-1-dependent fashion is an attractive hypothesis.

In the present study we utilize intact neutrophils and neutrophil cytoplasts (enucleate, granule-poor, metabolically active cell fragments) to demonstrate that MAPK activation by FMLP is associated with activation of p21^{ras} and translocation of Raf-1 to the PM and that cAMP acts via PKA to inhibit FMLP-stimulated MAPK activation in cytoplasts but not neutrophils. We also show that insulin, known to activate MAPK via p21^{ras} and Raf-1 in mitotic cells, activates MAPK in neutrophils and cytoplasts. Finally, we observed a strong correla-
tion between MAPK activation and cell-cell adhesion in neutrophils and cytoplasts suggesting a new regulatory role for MAPK in a process critical for inflammation.

**EXPERIMENTAL PROCEDURES**

Materials—Except where otherwise noted, reagents were purchased from Sigma. Accurep® was from Accurate Scientific, Inc. Dextran T500, acrylamide/bis solution, TEMED, and ammonium persulfate were from Pharmacia Biotech Inc. Human recombinant interleukin-8 (II-8) and myelin basic protein (MBP) substrate peptide (MBPp) were from Upstate Biotechnology Inc. MBP control peptide (valine substituted for threonine) was synthesized by Chiron Mimotopes. Anti-p44/42 monoclonal antibody Y13–259 was from Oncogene Science. Anti-Raf-1 monoclonal antibody was from Transduction Laboratories. Antisera specific for SOS1/2, Raf-1 (catalog sc-133), p44erk1 (sc-93), p42erk2 (sc-154), and p44erk1/p42erk2 (sc-94) were from Santa Cruz Biotechnology. Rabbit anti-mouse and rabbit anti-rat antisera were from Organon Teknika Corporation (Cappel), APT, GTP-S, and human recombinant insulin were from Boehringer Mannheim. [32P]ATP was from Amer- sham Corp. [125I]GTP was from DuPont NEN. 1:250-potassium A was from ICN. KT5720 was from Kamiya Biomedical Company. 5′-Ethylcarb oxamidoadenosine (NECA) was from Research Biochemicals Int. Anti-phosphotyrosine antibody was the kind gift of Dr. Benjamin Margolis. Anti-p44/42 antibody X-259 was from Oncogene Science. Anti-p44/42 monoclonal antibody Y13–259 was from Oncogene Science. Anti-Raf-1 monoclonal antibody Y13–259 was from Oncogene Science. Anti-Raf-1 monoclonal antibody Y13–259 was from Oncogene Science.
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Fig. 2. Anti-Erk antisera immunoprecipitate active and inactive p44erk and p42erk from cytoplast lysates. A, cytoplasts (1.5 × 10^6/condition) were incubated in the absence (lane 1) or presence of 100 nm FMLP (lanes 2 and 3) for 1 min at 37 °C, lysed, and immunoprecipitated with antisera to p44erk and p42erk together (lanes 1 and 2) or control antiserum (lane 3) as described under "Experimental Procedures." and then analyzed by SDS-polyacrylamide gel electrophoresis and Western blot with a third antibody directed against both p44erk and p42erk. B, cytoplasts (1.5 × 10^6/condition) were incubated in the absence (lane 1) or presence of 100 nm FMLP (lanes 2–5) for 1 min at 37 °C, lysed, and immunoprecipitated with antisera to p44erk and p42erk together (lanes 1 and 2), p44erk alone (lane 3), p42erk alone (lane 4), or control antiserum (lane 5) as described under "Experimental Procedures." and then analyzed by gel renaturation MAP kinase assay. Results shown are representative of four experiments.

Fig. 3. Kinetics of MAPK activation and p42erk phosphorylation/dephosphorylation in FMLP-stimulated cytoplasts. Cytoplasts stimulated with 100 nm FMLP at 37 °C for the times indicated were analyzed for MAP kinase activity by gel renaturation assay (A) and immunoblotting for phosphotyrosine-containing proteins (B) as described under "Experimental Procedures." C, the nitrocellulose from B was stripped (19) and reprobed with an anti-p44erk/p42erk antiserum. Results shown are representative of two experiments.

We confirmed the identity of cytoplasmic FMLP-sensitive MBP kinases as p44erk and p42erk by immunoprecipitating p44erk and p42erk from unstimulated or FMLP-stimulated cytoplasts and analyzing the precipitates by immunoblot and gel renaturation MAP kinase assays. Immunoprecipitation of p44erk and p42erk followed by immunoblot using a third antiserum recognizing both Erks revealed two polypeptides of expected molecular weight (Fig. 2A). Neither protein was precipitated by a control antiserum. In contrast, p44erk/p42erk antiserum precipitated MBP kinase activity in the 42–44-kDa region of the gel only from lysates of cytoplasts that had been stimulated with FMLP (Fig. 2B). The resolving power of the MBP-imregnated gels in these experiments was inadequate to distinguish p44erk from p42erk kinase activity. However, when p44erk and p42erk were immunoprecipitated separately from FMLP-stimulated lysates each precipitate contained 42–44-kDa MBP kinase activity, although somewhat less than the coprecipitate (Fig. 2B). FMLP-stimulated lysates immunoprecipitated with control antiserum contained no 42–44-kDa MBP kinase activity, although somewhat less than the coprecipitate (Fig. 2B).

Because neutrophils required post-stimulation processing that made precise kinetic measurements difficult, we studied the kinetics of FMLP-stimulated MAPK activation in cytoplasts that could be rapidly lysed in SDS sample buffer without releasing nucleic acids and granular proteases (Figs. 3A and 4). FMLP-stimulated activation of p44erk and p42erk was transient, peaking at 1 to 2 min and returning to baseline by 10 min. Because MAPK activity is associated with tyrosine phosphorylation of p44erk and p42erk, we compared MAPK kinase activities with tyrosine phosphorylation of cytoplasmic proteins following stimulation with FMLP (Fig. 3B). Cytoplast lysates contained a prominent 42-kDa tyrosine phosphoprotein whose phosphorylation was stimulated by FMLP with kinetics identical to those of the p42erk kinase activity. When the blot was stripped and reprobed with an antiserum directed to p44erk/p42erk (Fig. 3C), the 42-kDa phosphoprotein aligned precisely with p42erk. The amount of p44erk and p42erk detected by immunoblot did not change with FMLP stimulation, confirming that the antiphosphotyrosine blot detected changes in phosphotyrosine content, not amount of protein. Since the anti-Erk antiserum used has greater affinity for p44erk than p42erk, the immunoblot results revealing a darker p42erk band suggest that more p42erk than p44erk is expressed in human neutrophils. This could explain why the anti-phosphotyrosine antibody was apparently only sensitive enough to detect phosphorylated p42erk, a result consistent with earlier studies (27). These data confirm that enucleate neutrophil cytoplasts retain the signaling molecules necessary to respond to FMLP stimulation by phosphorylating and then dephosphorylating Erk on tyrosine and transiently activating MAPK activity.

Pathways of MAPK Activation in Neutrophils and Cytoplasts—G protein-stimulated MAPK activation has been
shown to proceed via both p21ras/Raf-1-dependent and independent pathways. We therefore studied whether MAPK activation by FMLP in neutrophils and cytoplasts was preceded by p21ras and Raf-1 activation. We first determined by immunoblot analysis that terminally differentiated neutrophils retain all of the elements of the classical p21ras/Raf-1-dependent MAPK pathway, including Grb2, SOS, Raf-1, MAPK or Erk kinase, and p44erk1 and p42erk2 in cytoplasts and p21ras in PM (not shown). To confirm that neutrophils retain the capacity to respond to stimuli that activate p21ras/Raf-1-dependent MAPK pathways, we examined the effect of PTKR agonists on neutrophils. Epidermal growth factor had no effect on neutrophil or cytoplast MAPK activity, suggesting a lack of expression of EGF receptor on myeloid cells. In contrast, insulin, at concentrations (20–200 nM) required to maximally activate MAPK in mitotic cells expressing insulin receptors (28), activated MAPK in neutrophils (312 ± 113% control) and cytoplasts (148 ± 21% control) with kinetics distinct from those of FMLP stimulation (Fig. 4). Thus at least one PTKR known to activate MAPK in a p21ras/Raf-1-dependent fashion is functionally expressed in human neutrophils suggesting that p21ras/Raf-1 signalling is intact.

To study the rapid FMLP-stimulated p21ras activation predicted by the kinetics of MAPK activation, we studied FMLP-stimulated guanine nucleotide exchange on p21ras. Because isolated neutrophils had a bench life (<6 h by lactate dehydrogenase release assay) inadequate to ensure equilibrium labeling of nucleotide pools by metabolic labeling with [32P]orthophosphate, we permeabilized neutrophils by electroporation in the presence of [α-32P]GTP to rapidly label intracellular pools of GTP. In this system p21ras activation is measured as total [α-32P]guanine nucleotide loading (guanine nucleotide exchange with or without GTPase activation). Electroporated neutrophils were stimulated with FMLP (100 nM), p21ras was immunoprecipitated from cell lysates, and the amount of [α-32P]guanine nucleotide associated with p21ras was determined by thin layer chromatography (Table I). The amount of [α-32P]guanine nucleotide associated with p21ras following FMLP stimulation was 164 ± 20% control at 30 s and remained stable for as long as 5 min, suggesting that FMLP-stimulated guanine nucleotide exchange on p21ras in neutrophils peaks no later than 30 s after stimulation. Since hydrolysis of [α-32P]GTP on p21ras results in p21ras [α-32P]GDP, total [α-32P]guanine nucleotide associated with p21ras cannot distinguish transient from persistent p21ras activation. However, the percentage of [α-32P]guanine nucleotide associated with p21ras as [α-32P]GTP declined after 1 min, suggesting a GTPase-activating protein activity limiting activation.

Membrane association of Raf-1 appears to be required for its activation by PTKR (7–9). Since Raf-1 is recruited to the PM of cells transfected with oncogenic, activated p21ras (7, 9), we tested whether FMLP, in addition to activating p21ras, can stimulate Raf-1 translocation from the CS to PM of cytoplasts. When unstimulated cytoplasts were sonicated and separated by centrifugation into soluble and insoluble fractions, 14 ± 3% of total immunodetected Raf-1 (supernant + pellet) was associated with the pellet. This analysis is likely to overestimate the true membrane-associated pool since cytoplast disruption may not have been complete and vesiculated cytoplast membrane is likely to sequester CS. FMLP stimulated a 2.2-fold increase in the amount of Raf-1 associated with cytoplast membranes. Thus, despite the potential overestimation of basal membrane-associated Raf-1, our system was sensitive enough to detect membrane translocation of this molecule. Kinetic analysis (Fig. 5) revealed that Raf-1 translocation to the membrane peaked at 30 s to 1 min and remained stable for at least 10 min following FMLP stimulation. In contrast to Raf-1, we observed no FMLP-stimulated translocation of p44erk1 or p42erk2 to PM in cytoplasts. Although neutrophil CS contained an abundant supply of SOS, this molecule also did not translocate from cytoplast CS to PM in response to FMLP. Thus, both FMLP-stimulated p21ras activation and Raf-1 translocation preceded MAPK activation, consistent with a role for both events upstream of Erk activation in the FMLP-stimulated pathway.

PTKR activation of MAPK via p21ras and Raf-1 may be down-regulated by cAMP-dependent, PKA-mediated phosphorylation of Raf-1, resulting in impaired interactions between p21ras and Raf-1 (12, 13). We therefore tested whether FMLP-stimulated MAPK activity in neutrophil cytoplasts can be similarly inhibited by cAMP. The membrane-permeable, phosphodiesterase-resistant CAMP analog dibutyryl CAMP (Bt2CAMP) (1 μM) completely inhibited insulin-stimulated cytoplast MAPK activity and inhibited FMLP-stimulated cytoplast MAPK activity by 46.4 ± 11.7% (Fig. 6A). Agents that raise intracellular cAMP by indirect mechanisms, including isobutylmethylxanthine (50 μM), forskolin (50 μM), isoproterenol (10 μM), and the adenosine A2 receptor agonist NECA (10 μM) also inhibited FMLP-stimulated MAPK activity by approximately 50% (Fig. 6B). To confirm that CAMP inhibition of FMLP-stimulated MAPK in cytoplasts is PKA-dependent, we tested the effect of KT5720 that, at concentrations below 2 μM, is a specific inhibitor of PKA (29). KT5720 (1 μM) reversed the inhibitory effect of Bt2CAMP on FMLP-stimulated MAPK activation (6C). These data

| Duration of FMLP stimulation | Nucleotide exchange (% control) | GTP-associated (% control) |
|-----------------------------|---------------------------------|---------------------------|
| 0 s                         | 100                             | 7 ± 3                     |
| 30 s                        | 164 ± 20                        | 18 ± 4                    |
| 1 min                       | 136 ± 17                        | 13 ± 4                    |
| 5 min                       | 133 ± 32                        | 4 ± 3                     |
bated for 10 min with 1 mM Bt2cAMP, stimulated with 100 nM FMLP for the times indicated, sonicated, and separated into membrane and soluble fractions as described under "Experimental Procedures." Fractions were assayed for Raf-1 by immunoblot quantitated by phosphorimaging. Results shown are the mean ± S.E. for three or more experiments.

Yu et al. (37) have recently reported that cAMP failed to inhibit FMLP-stimulated MAPK activity in human neutrophils. To explore this discrepancy we compared the effect of Bt2cAMP on FMLP-stimulated MAPK activity in neutrophils and cytoplasts (Fig. 6D). As measured by the MBPp kinase activity assay, 1 mM Bt2cAMP inhibited FMLP-stimulated cytoplast MAPK activity by 53 ± 13% but had no effect on FMLP-stimulated MAPK activity in neutrophils. Thus, a regulatory role for cAMP in MAPK activation can be observed in vitro in lysates from cytoplasts but not from intact neutrophils suggesting that a factor(s) derived from the nucleus and/or cytoplasmic granules masks the effect.

Role of MAPK in Neutrophil Homotypic Aggregation—To determine a potential role for MAPK activation in neutrophil function, we compared MAPK activation with neutrophil adhesiveness as measured by homotypic aggregation in neutrophils and cytoplasts. Although each of the best-studied neutrophil chemoattractants (FMLP, LTB4, C5a, and IL-8) acts through a similar seven transmembrane-spanning receptor linked to a similar or identical G protein (likely Gi2), their efficacies at stimulating neutrophil functions vary dramatically. We therefore compared the ability of each chemoattractant to stimulate MAPK activity with its effect on aggregation. Like FMLP, saturating concentrations of LTB4, C5a, and IL-8 stimulated MAPK activity in intact neutrophils by 1 min (Table II); however, LTB4, C5a, and IL-8 were significantly less efficacious than FMLP in activating MAPK. The extent of aggregation stimulated by each agonist correlated well (R2 = 0.92) with the degree of MAPK activation (Fig. 7, A and C). LPA, which acts via a G protein-linked receptor and p21ras to stimulate MAPK in cells in culture (14), had no effect on neutrophil MAPK activity or homotypic aggregation (not shown). In cytoplasts, whereas FMLP stimulated MAPK activity (318 ± 34% control), the other chemoattractants demonstrated little or no effect on MAPK activity (Table II). As in intact neutrophils, the ability of the chemoattractants to stimulate cytoplast aggregation correlated well (R2 = 0.99) with their ability to stimulate cytoplast MAPK activity (Fig. 7, B and D). These data confirm divergent signaling from distinct G protein-linked receptors and argue strongly for an association between MAPK activation and adhesion. The ability of Bt2cAMP to inhibit FMLP-stimulated MAPK activity in cytoplasts but not neutrophils suggested that similar effects might be observed on homotypic aggregation. Indeed, Bt2cAMP (1 mM) had no effect on FMLP-stimulated neutrophil aggregation but inhibited FMLP-stimulated cytoplast aggregation by 33 ± 2% (Fig. 8). This observation further supports a role for MAPK activation in neutrophil adhesion.

In contrast to the chemoattractants, insulin activated neutrophil and cytoplasm MAPK but failed to stimulate neutrophil aggregation, suggesting that MAPK activation may be necessary but not sufficient to support aggregation. We therefore tested whether insulin could prime neutrophils for chemoat-

![FIG. 5.](image)

**FIG. 5.** FMLP stimulates translocation of Raf-1 from CS to PM in cytoplasts. Cytoplasts were incubated at 37°C in the absence or presence of 100 nM FMLP for the times indicated, sonicated, and separated into membrane and soluble fractions as described under "Experimental Procedures." Fractions were assayed for Raf-1 by immunoblot quantitated by phosphorimaging. Results shown are the mean ± S.E. for four experiments.

![FIG. 6.](image)

**FIG. 6.** cAMP inhibits FMLP-stimulated MAPK activity in cytoplasts but not neutrophils. A, cytoplasts were incubated for 5 min in the absence or presence of 1 mM Bt2cAMP, stimulated (100 nM FMLP for 1 min or 200 nL insulin for 10 min), and analyzed for MAP kinase activity by gel renaturation assay quantitated by phosphorimaging. B, cytoplasts were incubated for 5 min in the presence of isobutylmethyloxanthine (50 μM), forskolin (50 μM), isoproterenol (10 μM), or NECA (10 μM) and stimulated for 1 min with 100 nM FMLP and analyzed by gel renaturation assay and phosphorimaging. C, cytoplasts were incubated for 5 min in the absence or presence of the specific PKA inhibitor KT5720 (1 μM), followed by 5-min incubation with Bt2cAMP (1 mM) and 1 min stimulation with 100 nM FMLP, and analyzed by gel renaturation assay and phosphorimaging. D, neutrophils or cytoplasts were incubated for 10 min with 1 mM Bt2cAMP, stimulated with 100 nM FMLP for 1 min, and analyzed for MBPp kinase activity. Results are expressed as the percent of stimulated MAPK activity in the absence of drugs and are given as the mean ± S.E. of three experiments.
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FIG. 7. Chemoattractant-induced homotypic aggregation and MAPK activation in neutrophils and cytoplasts. Neutrophils (10⁶/ml) (A) and cytoplasts (5 × 10⁷/ml) (B) prepared from the same donor were analyzed for homotypic aggregation in response to FMLP (100 nM), LTB₄ (300 nM), C5a (100 nM), and IL-8 (100 nM). The correlations between MAPK activation (abscissae) and aggregation (ordinate) in neutrophils (C) and cytoplasts (D) in response to various chemoattractants were plotted, and regression coefficients were calculated. Results shown are representative (A and B) or the means (C and D) of three experiments.

FIG. 8. Bt-cAMP inhibits FMLP-stimulated homotypic aggregation in cytoplasts but not in neutrophils. Neutrophils or cytoplasts were incubated for 15 min at 37 °C in the absence or presence of 1 mM Bt-cAMP and then assayed for homotypic aggregation in response to 100 nM FMLP. Results shown are the means ± S.E. for three or more experiments.

Although well established, the link between chemoattractant-stimulated G protein signaling pathways and the MAPK cascade is poorly elucidated. Neutrophils are a good system in which to study G protein-mediated signaling because the cellular responses are rapid and easily quantitated. Enucleate, granule-depleted neutrophil cytoplasts retain the capacity to respond to chemoattractants (30) and thus represent a simplified system useful in studying chemoattractant signaling through G proteins. We employed neutrophils and cytoplasts to study the kinetics of chemoattractant-stimulated activation of p21ras, Raf-1, and MAPK and observed an association between MAPK activation and cell-cell adhesion.

The analysis of MAPK activity in cytoplasmic lysates by an MBP kinase gel renaturation assay offered distinct advantages over similar studies of lysates of intact neutrophils, including resolution of two MAPKs in cytoplasmic lysates, identified by immunoprecipitation as p44erk1 and p42erk2. Moreover, the ability to terminate stimulation by direct addition of SDS sample buffer permitted more accurate kinetic analysis of cytoplasts than of neutrophils. FMLP-stimulated cytoplast MAPK activation was rapid and transient, consistent with a role for MAPK in signaling pathways for neutrophil functions such as O₂⁻ generation, degranulation, and cell-cell adhesion but slower than the previously reported kinetics of neutrophil MAPK activation (19, 27, 31). The greater precision afforded by kinetic analysis of MAPK in cytoplasts thus allowed comparison with the kinetics of activation of other putative elements in the FMLP-stimulated MAPK cascade, such as p21ras and Raf-1. The use of cytoplasts also permitted observation of MAPK signaling in the absence of nuclear or granular elements. Thus, phosphorylation and dephosphorylation on tyrosine residues of p42erk2 in cytoplasts, with kinetics paralleling those of MAPK activation, indicate that the molecular machinery required for...
regulating MAPK activity by Erk kinase and phosphatase activities is retained in cytoplasts and so independent of any nuclear factors that may regulate MAPK. This observation may distinguish neutrophils from proliferating cells, in which activated MAPK translocates to the nucleus where it is downregulated by dual phosphothreonine/phosphotyrosine phosphatases such as PAG-1 (32).

Although a wide variety of G protein-linked and non-G protein-linked receptors have been demonstrated on neutrophils, none of the classical PTKRs have been reported. Insulin, however, has been shown to bind to human neutrophils (33), stimulate chemokinesis (34), and prime for chemotaxis to FMLP (35), indicating that PTKRs for insulin are expressed on these cells. Our observation that insulin activated MAPK in neutrophils and cytoplasts suggests that a p21ras/Raf-1 pathway is functionally intact and can be engaged by at least one PTKR. Thus, the neutrophil formyl peptide receptor may also activate MAPK through the p21ras/Raf-1 pathway. However, the longer latency for insulin- than for FMLP-stimulated MAPK activation indicates that the pathways to p21ras activation may be distinct.

Our observation that FMLP activated p21ras in neutrophils supports p21ras/Raf-1 signaling. Although a previous study came to the same conclusion using neutrophils metabolically labeled with [32P]orthophosphate (36), we found the bench life of neutrophils insufficient to label nucleotide pools to equilibrium, an absolute requirement for interpreting GTP/GDP ratios of GTase-bound nucleotide as an indicator of p21ras activation. We therefore analyzed total labeled guanine nucleotide associated with immunoprecipitated p21ras from lysates of cells electrophorosed in the presence of [alpha-32P]GTP and found maximal increase after 30 min of exposure to FMLP (i.e. preceding peak MAPK activity). Concordant with a prior report (36), the proportion of [alpha-32P]GTP associated with p21ras declined by 5 min, suggesting sequential guanine nucleotide exchange factor and GTase-activating protein activities following FMLP stimulation.

Raf-1 has been shown to translocate from CS to PM in cultured cells exposed to serum (7). However, Raf-1 translocation in response to ligation of neither a specific PTKR nor a G protein-linked receptor has been demonstrated in any cell type. We have shown that cytoplasts are an ideal system with which to assay PM translocation of cytosolic proteins (24). Using this system, we now report FMLP-stimulated translocation of Raf-1 to the PM. These data complement those of Worthen et al. (36) who reported FMLP-stimulated Raf-1 kinase activity. Like p21ras activation, FMLP-stimulated Raf-1 translocation preceded MAPK activation. Our failure to observe SOS translocation in response to FMLP suggests that SOS may not participate in G protein activation of p21ras. Alternatively, translocation of SOS may not be necessary for its activity, or the kinetics of SOS translocation may be too rapid to have been appreciated in our assay.

Our observation that MAPK activity in cytoplasts was inhibited by agents that raise intracellular cAMP and that a PKA antagonist reversed this inhibition is also consistent with p21ras/Raf-1-dependent signaling since cAMP has been shown to down-regulate MAPK activation by PKA-dependent phosphorylation of Raf-1, inhibiting p21ras/Raf-1 interactions (12, 13). Bt2cAMP has been shown to inhibit neutrophil Raf-1 kinase activity (36), supporting an effect of cAMP in neutrophils at the level of Raf-1. However, Bt2cAMP inhibition of FMLP-stimulated MAPK activation has not previously been demonstrated. Indeed, Yu et al. (37) reported that Bt2cAMP does not inhibit FMLP-stimulated MAPK in cytocatalasin B-treated neutrophils. Our data confirm this observation in intact neutrophils but show that cytoplasts express a cAMP-sensitive pathway. The exposure of a cAMP-sensitive pathway in cytoplasts may be explained by increased phosphodiesterase or Raf-1 phosphatase activities in detergent lysates of granule-replete, nucleated neutrophils. Alternatively, neutrophils may possess both cAMP-sensitive and -insensitive G protein-linked pathways of MAPK activation, the latter preferentially inactivated during cytoplast preparation.

Several groups have proposed a role for MAPK in neutrophil O2 generation (39). However, Yu et al. (37) have recently demonstrated that MAPK activation and O2 generation can be dissociated in neutrophils. In contrast, we observed a good correlation between MAPK activation and cell-cell adhesion in both neutrophils and cytoplasts. The extent of MAPK activation correlated closely with the degree of homotypic aggregation stimulated by each of four chemoattractants in both cells and cytoplasts. Interestingly, both cell-cell adhesion and MAPK responsiveness to LTB4, C5a, and IL-18 were preferentially lost in the process of preparing cytoplasts relative to their responsiveness toward FMLP. Whereas Yu et al. (37) observed a discordance between the marked inhibition of O2 generation by Bt2cAMP (37, 40) and its failure to inhibit MAPK activation in FMLP-stimulated neutrophils (37), in our studies Bt2cAMP inhibited neither FMLP-stimulated MAPK activation nor FMLP-stimulated homotypic aggregation in neutrophils but significantly inhibited both of these responses in cytoplasts. Thus the effect of Bt2cAMP on FMLP-stimulated MAPK activity in both neutrophils and cytoplasts correlated with its effect on cell-cell adhesion. Our discovery that insulin both activated MAPK in human neutrophils and primed these cells for homotypic aggregation in response to chemoattractants demonstrates a further correlation between MAPK activation and cell-cell adhesion. The inability of insulin to directly stimulate aggregation suggests that MAPK may be necessary but not sufficient to directly or indirectly regulate adhesion molecules. The failure of insulin to stimulate or prime neutrophils for O2 generation or degranulation in the absence of extracellular glucose suggests the hypothesis that MAPK regulates some but not all neutrophil functions. Thus, although O2 generation can be dissociated from MAPK activation, our studies of neutrophils and cytoplasts support a role for MAPK activation in cell-cell adhesion.

Neutrophil homotypic aggregation is mediated by activation of the beta2 integrin CD11b/CD18 (25). The activation states of integrins appear to be regulated by interactions of the cytoplasmic domains of these heterodimeric transmembrane glycoproteins with the actin cytoskeleton through focal adhesion plaques (41). Thus, MAPK might regulate cell-cell adhesion through phosphorylation of molecules regulating focal adhesion plaques. In addition to a hypothetical role in regulating the actin cytoskeleton, MAPK's have a well-established role in regulating the microtubule cytoskeleton by associating with and phosphorylating microtubule-associated proteins (42). The relationship between MAPK activation and leukocyte adhesion suggests new targets for anti-inflammatory drugs since leukocyte adhesion to vascular endothelium is the first committed step in the inflammatory response. Furthermore, insulin stimulation of neutrophil MAPK and priming for chemoattractant-stimulated adhesion suggest a molecular mechanism for impaired neutrophil function in type 1 diabetes, a state of insulin deficiency associated with increased susceptibility to bacterial infection.
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Since neutrophils are terminally differentiated, non-mitotic cells, the effects of MAPK on transcription factors related to growth and differentiation are unlikely to be relevant. Our studies with enucleate cytoplasts support this view. Marshall (43) has recently proposed that the outcome of MAPK signaling is dependent largely on its duration of activation. If so, rapid MAPK activation in neutrophils may represent a distinct category of signaling. Alternatively, differentiated cells might also be distinguished by their complement of MAPK substrates. The well-defined MAPK substrate also implicated in neutrophil activation is cytoplasmic phospholipase A2 (44). Further studies are likely to identify other MAPK substrates involved in rapid neutrophil responses.

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