Communication

Binding of Receptor-recognized Forms of α2-Macroglobulin to the α2-Macroglobulin Signaling Receptor Activates Phosphatidylinositol 3-Kinase*

(Received for publication, February 24, 1998, and in revised form, April 1, 1998)

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Ligation of the α2-macroglobulin (α2M) signaling receptor by receptor-recognized forms of α2M (α2M*) initiates mitogenesis secondary to increased intracellular Ca2+. We report here that ligation of the α2M signaling receptor also causes a 1.5–2.5-fold increase in wortmannin-sensitive phosphatidylinositol 3-kinase (PI3K) activity as measured by the quantitation of phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 formation was α2M*-dependent concentration-dependent with a maximal response at ~50 μM ligand concentration. The peak formation of PIP3 occurred at 10 min of incubation. The α2M receptor binding fragment mutant K1370R which binds to the α2M signaling receptor activating the signaling cascade, increased PIP3 formation by 2-fold. The mutant K1374A, which binds very poorly to the α2M signaling receptor, did not cause any increase in PIP3 formation. α2M*-induced DNA synthesis was inhibited by wortmannin. 1,2-Bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid toxymethylester a chelator of intracellular Ca2+, drastically reduced α2M*-induced increases in PIP3 formation. We conclude that PI3K is involved in α2M*-induced mitogenesis in macrophages and intracellular Ca2+ plays a role in PI3K activation.

The α-macroglobulins are part of a large super family including human α2-macroglobulin (α2M) (1, 2). Proteolytic attack on the bait region or direct nucleophilic attack on the thiolester bonds of human α2M subunits triggers a major conformational change that exposes receptor recognition sites present in each of the four α2M subunits (2, 3). Two receptors bind α2M*, namely, LRP/α2MR and a recently discovered α2M signaling receptor (α2MSR) (4–13). LRP/α2MR is a scavenger receptor that binds a wide variety of ligands. Binding of α2M* to LRP/α2MR is followed by uptake and degradation in lysosomes but not activation of a signaling cascade (7, 8, 12). By contrast, binding of α2M* or RBF to α2MSR triggers classical signaling cascades and regulates cellular proliferation (6–14).

The agonist-induced entry of Ca2+ from the extracellular medium is of major importance in the cytosolic Ca2+ signals that link activation of various receptors on the cell surface with the initiation and control of cell functions (15–17). Elevated cytosolic Ca2+ modulates specific cell cycle events and DNA synthesis (18–23). Binding of α2M* to α2MSR raises p21CAF and α2M*-induced increases in p21ASGTP levels 2–3-fold in macrophages and pretreatment with wortmannin, a specific inhibitor of PI3K, does not affect α2M*-induced increases in p21ASGTP levels (24).

Cellular 3-phosphoinositides are generated through the action of a family of PI3Ks (25, 26). PI3K activity was first reported in association with v-SRC and v-RAS oncogenes, where it catalyzes phosphorylation of inositol at the D-3 position of phosphatidylinositol (PI(3,4,5)). Phosphatidylinositol 3,4,5-trisphosphate (PIP3) is observed in several cell types on stimulation with growth factors, cytokines, insulin, f-Met-Leu-Phe, and platelet-activating factor, RAS, and viral transformation (25–27). Signaling by 3-phosphoinositides regulates diverse functions such as mitogenesis, cell growth, membrane ruffling, chemotaxis, oxidant production, secretory responses, insulin-mediated membrane translocation of the glucose transporter, membrane trafficking of growth factor receptors, cell adhesion, and Na+/H+ exchange (25–27). Since many of the cellular responses elicited upon ligation of α2MSR with receptor-recognized forms of α2M are similar to those elicited upon binding of growth factors to their receptors, we studied the activity of PI3K by measuring the formation of PtdIns 3,4,5-trisphosphate (PIP3), in murine macrophages stimulated with α2M*. Ligation of α2MSR increases the wortmannin-sensitive formation of PIP3 2–3-fold in a concentration-dependent manner and that the agonist-induced formation of PIP3 is influenced by [Ca2+]i levels.

EXPERIMENTAL PROCEDURES

Materials—Human α2M, α2M-methylamine (α2M*), RBF and its mutants K1370A and K1374R were prepared as described (13). The sources of thiglycollate-elicited macrophages and cell culture requirements have been described previously (7–10). PtdIns 4-phosphate (P(4)IP), PtdIns 4,5-bisphosphate (P(4,5)IP) and PtdIns 3,4,5-trisphosphate (PIP3) were from Biomol (Plymouth Meeting, PA). Insulin, wortmannin, thapsigargin, fatty acid-free bovine serum albumin (BSA) and molybdenum blue spray were from Sigma. Fura 2/AM and BAPTA/AM were from Molecular Probes (Eugene, OR). [3H]Thymidine (specific activity 70 Ci/mmol) and [3H]myoinositol (specific activity 20 Ci/mmol) were from American Radiolabeled Chemicals (St. Louis, MO). Silicone gel G plates were from Analtech (Dover, DE). All other reagents used were of analytical grade.

Measurement of PtdIns 3,4,5-Trisphosphate Formation—PI(3,4,5)IP is
tion in murine peritoneal macrophages was measured essentially according to the method of Okada et al. (29) except that [3H]myo-inositol was used to label inositol lipids in place of [32P]. Briefly thiglycollate-elicited macrophages (~8 × 10^6/well) were collected in Hanks' balanced salt solution (HBSS) containing 10 mM HEPES, pH 7.4, and were allowed to adhere for 2 h in RPMI 1640 medium containing 2 mM glutamine, 12.5 units of penicillin/ml, and 6 μg of streptomycin/ml, and 5% fetal bovine serum at 37 °C in a humidified CO_2 (5%) incubator. Nonadherent cells were removed with cold HBSS, and a volume of RPMI 1640 medium was added containing the additions listed above except that 0.2% fatty acid-free BSA was substituted for the serum. To each well 5 μCi/ml [3H]myo-inositol, 30 μg/ml, was added, and the cells were incubated as above for 20 h. The monolayers were washed four times with the above RPMI 1640 medium, a volume of the medium added to each well, and the cells preincubated for 3 min at 37 °C before stimulation with different agonists for 10 min. In experiments where the effect of wortmannin on agonist-induced formation of PIP_3 was studied, it was incubated (30 min) with samples for 30 min at 37 °C to prior to addition of agonists. In experiments where the effects of modulation of intracellular Ca^{2+} by thapsigargin (100 nM) and BA-PAT/AM (10 μM) were to be studied on PIP_3 formation, the former was added 10 min and the latter 30 min before the addition of the agonist. The reaction was terminated by aspirating the medium, a volume of chilled methanol was added to each well, and the lipids were extracted and separated on oxalate-impregnated silica gel G plates as described by Okada et al. (29). Authentic standards of PIP, PIP_2, and PIP_3 were co-chromatographed with each run. The chromatoplates were air-dried and phospholipid spots detected by lightly spraying with molydenum blue spray (30). The R_f values obtained under the experimental conditions for PIP, PIP_2, and PIP_3 were 0.63, 0.23, and 0.12, respectively. Gel areas corresponding in R_f values to PIP, were scraped into scintillation vials and the radioactivity determined by liquid scintillation counting. In preliminary experiments, the identity of [3H]-labeled PIP, PIP_2, and PIP_3 on the chromatoplates was established by autoradiography of developed chromatoplates on Kodak BioMax film using BioMax TranScreen-LE intensifying screen (Eastman Kodak Co.) at −70 °C for 10 days and comparing the R_f values of radioactive spots with authentic standards co-chromatographed and (30) by adding authentic standards of PIP, PIP_2, and PIP_3 (15 μg each) to samples prior to chromatography and spraying the developed chromatoplates with molydenum blue spray (30).

**Measurement of DNA Synthesis**—DNA synthesis was measured according to Charlesworth and Rozenzweig (11, 23). Briefly, 2-h adhered macrophages (4 × 10^5 cells/well) were incubated in a volume of RPMI 1640 medium containing glutamine, penicillin, streptomycin, and 0.2% fatty acid-free BSA. To each well 1 μCi/ml [3H]thymidine (2 μCi/ml) was added followed by the addition of different ligands to the respective wells and the incubations continued as above for 20 h. In experiments where the effects of wortmannin (30 nM) with samples for 30 min at 37 °C prior to addition of agonists. In experiments where the effects of modulation of intracellular Ca^{2+} by thapsigargin (100 nM) and BA-PAT/AM (10 μM) were to be studied on PIP_3 formation, the former was added 10 min and the latter 30 min before the addition of the agonist. The reaction was terminated by aspirating the medium, a volume of chilled methanol was added to each well, and the lipids were extracted and separated on oxalate-impregnated silica gel G plates as described by Okada et al. (29). Authentic standards of PIP, PIP_2, and PIP_3 were co-chromatographed with each run. The chromatoplates were air-dried and phospholipid spots detected by lightly spraying with molydenum blue spray (30). The R_f values obtained under the experimental conditions for PIP, PIP_2, and PIP_3 were 0.63, 0.23, and 0.12, respectively. Gel areas corresponding in R_f values to PIP, were scraped into scintillation vials and the radioactivity determined by liquid scintillation counting. In preliminary experiments, the identity of [3H]-labeled PIP, PIP_2, and PIP_3 on the chromatoplates was established by autoradiography of developed chromatoplates on Kodak BioMax film using BioMax TranScreen-LE intensifying screen (Eastman Kodak Co.) at −70 °C for 10 days and comparing the R_f values of radioactive spots with authentic standards co-chromatographed and (30) by adding authentic standards of PIP, PIP_2, and PIP_3 (15 μg each) to samples prior to chromatography and spraying the developed chromatoplates with molydenum blue spray (30).

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**RESULTS AND DISCUSSION**

α MSR Ligation with α MSR Increases PIP_3 Levels—The effect of α MSR on the synthesis of PIP_3 in macrophages is shown in Fig. 1. The maximum synthesis of PIP_3 occurred at a ligand concentration of 50-500 nM (5A). The kinetics of PIP_3 formation is similar to that noted previously for p21^{RAS}-stimulated (24) in macrophages stimulated with α MSR. Since wortmannin treatment had no effect on α MSR-stimulated p21^{RAS} synthesis (24), PI3K is downstream of RAS, consistent with the report that PI3K is a substrate for activated RAS (32). The synthesis of PIP_3 stimulated with 100 μM of α MSR was maximal after a 10-min period of incubation but declined at longer periods of incubations (Fig. 1B). The α MSR-induced synthesis of PIP_3 was comparable with the effect of insulin (20 nM) (Fig. 2A), a potent activator of PI3K (33). That the increase in PIP_3 formation occurs due to the binding of α MSR to α MSR was confirmed by using a RBF of α MSR and its mutants (28). Both RBF and its mutant K1370R, which bind to α MSR and generate signaling events similar to that of α MSR (33), caused a 2-fold increase in PIP_3 synthesis (Fig. 2B). By contrast, the binding site mutant K1374A, which binds poorly to α MSR, does not elicit increases in IP_3 formation or increases in [Ca^{2+}](33) and failed to stimulate PIP_3 synthesis (Fig. 2B). Wortmannin, a potent and specific inhibitor PI3K activity (34), completely inhibited α MSR-, RBF-, and insulin-induced increases in PIP_3 synthesis (5A and 2). We also tested the effect of 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002) on α MSR-induced PIP_3 synthesis. LY294002 is a specific inhibitor PI3K, albeit its EC_{50} is greater than wortmannin (35, 36). This inhibitor almost completely abolished PIP_3 synthesis in macrophages exposed to α MSR (Table 1).

**Ligation of α MSR with α MSR in cells with activation of PLCγ (9), increases in intracellular pH (9), activation of PLA_2 and PLD (37), synthesis and secretion of PAF (38) and PGE_2 (39), increases in p21^{RAS} levels (24), and mitogenesis (10, 11, 24). The present studies in conjunction with these previous observations show that like established tyrosine kinase receptors, cellular responses elicited upon ligation of α MSR involve several signaling cascades, including p21^{RAS}, PI3K, and mitogen-activated protein kinase (MAPK) pathways.

**Wortmannin Inhibits RBF-induced DNA Synthesis**—A num-
The number of signal transduction pathways have been implicated in regulating cell growth and differentiation in response to G-protein-coupled receptor agonists that activate protein tyrosine kinase receptors (see Ref. 40 for review). These pathways include cascades involving the Ser/Thr kinase families, MAPK, and the ribosomal S6 kinases (25, 26, 28–41). PI3K has been implicated in the regulation of cell growth in a variety of cell types (25, 26, 42). The lipid product of PI3K is not broken down by phospholipase C but seems to act as second messenger playing a role in Ca2+ mobilization, actin arrangement, and activation of Ser/Thr kinases such as isoforms of PKC and

**TABLE I**

| Addition | PIP3 synthesis (pmol/10^6 cells) |
|----------|----------------------------------|
| None     | 63.17 ± 0.90a                   |
| LY294002 alone | 74.00 ± 3.00            |
| α-M* (100 pm) | 158.10 ± 4.06            |
| LY294002 (20 µM) + α-M* | 69.82 ± 0.00            |

Values are the mean ± S.E. from two separate experiments.

**TABLE II**

| Addition | DNA synthesized (fmol/mg protein) |
|----------|----------------------------------|
| None     | 473.32 ± 32.00a                   |
| RBF (100 pm) | 1147.70 ± 144.00                |
| Wortmannin (30 nm/30 min) + RBF | 542.71 ± 48.00                 |

Values are mean ± S.E. from two independent experiments performed in quadruplicates.
protein kinase B (PKB also known as cAKT). The latter are activated consequent to PI3K activation in cells treated with growth factors and mitogens, overexpression of PI3K, and inhibited by wortmannin and by dominant negative subunit mutants of PI3K (25–27, 41). Downstream targets of PKB include p70 ribosomal kinase associated with up-regulation of transcripts for ribosomal proteins and elongation factors (43, 44).

We have assessed the involvement of PI3K in RBF-induced DNA synthesis in macrophages by using wortmannin (Fig. 3A). Incubation of cells with wortmannin (30 nM/30 min/37 °C) prior to stimulation with RBF (100 pM) nearly abolished RBF-induced DNA synthesis (Table II), which shows that the PI3K signaling pathway is involved in DNA synthesis in cells stimulated with receptor-recognized forms of αM. The PI3K inhibitor LY294002 also nearly abolished DNA synthesis induced by RBF or αM* (data not shown).

**Chelation of [Ca2+]i with BA**P**TA/AM Inhibits αM*-induced PIP2 Synthesis.—We have previously reported the dependence of protein and DNA synthesis on intracellular Ca2+ levels in macrophages stimulated with αM* (11). We have now examined the role of [Ca2+]i on PIP2 synthesis in macrophages stimulated with αM* in several ways: 1) by modulating [Ca2+]i, with thapsigargin (100 nM/10 min/37 °C), an endoplasmic reticulum Ca2+-ATPase inhibitor that raises [Ca2+]i, by releasing Ca2+ from both IP3-dependent and IP2-independent internal Ca2+ pools and 2) by use of BAPTA/AM (10 mM/30 min/37 °C) that chelates [Ca2+]i. Thapsigargin alone increased PIP2 synthesis comparable with that seen with αM* or with thapsigargin plus αM* (Fig. 2C). By contrast, BA**P**TA/AM nearly abolished αM*-induced PIP2 synthesis (Fig. 2C). We have reported previously that manipulating IP2 and [Ca2+]i, profoundly alters agonist-induced increases in protein and DNA synthesis (10, 11). In light of the importance of [Ca2+]i in αM*-induced DNA synthesis, and inhibition of DNA synthesis by wortmannin (Fig. 2C) we evaluated the effect of wortmannin on αM*-induced synthesis of IP2 and changes in [Ca2+]i (Fig. 3). Wortmannin by itself showed no effect on IP3 synthesis in macrophages, and when administered before αM*, it only slightly attenuated IP3 synthesis (about 10–15%) compared with αM*-treated cells (Fig. 3A). As expected, treatment of cells with wortmannin before αM* only slightly attenuated both the IP3-induced increase in [Ca2+]i, as well as Ca2+ entry from the medium (Fig. 3B).

The tyrosine kinase class of receptors, which include growth factor receptors, upon activation, induce mitogenesis via a series of downstream steps that may show cellular variance and include signaling proteins Grb2, Sos, Ras, Raf, MEK, and MAPK (40). We show here, for the first time, that insulin and other growth factors, receptor-recognized forms of αM, upon binding to the α2MSR, also induce the activation of wortmannin-sensitive PI3K. Thus as suggested earlier (10–14), in addition to being a classical endocytic receptor, α2MSR also appears to have an additional role in tissue repair.