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Pertussis toxin-sensitive activation of p21^{ras} by G protein-coupled receptor agonists in fibroblasts

(thrombin/lysophosphatidic acid/epidermal growth factor/signal transduction)

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ABSTRACT **Some agonists of G protein-coupled receptors, such as thrombin and lysophosphatidic acid (LPA), can promote cell proliferation via a pertussis toxin (PTX)-sensitive signaling pathway. While these agonists stimulate phospholipase C and inhibit adenylate cyclase, it appears that other, as-yet-unidentified, effector pathways are required for mitogenesis. Here we report that LPA and a thrombin receptor agonist peptide rapidly activate the protooncogene product p21^{ras} in quiescent fibroblasts. This activation is inhibited by PTX and yet not attributable to known PTX-sensitive G protein pathways, including stimulation of phospholipases, inhibition of adenylate cyclase, or modulation of ion channels. LPA- and peptide-induced p21^{ras} activation is inhibited by the tyrosine kinase inhibitor genistein, at doses that do not affect epidermal growth factor-induced p21^{ras} activation. Thus, a heterotrimeric G protein of the G_i subfamily regulates activation of p21^{ras} by LPA and thrombin, possibly through an intermediary tyrosine kinase. This pathway may critically participate in mitogenic signaling downstream from certain G protein-coupled receptors.**

The signaling mechanisms by which growth factors stimulate cell proliferation have not been fully identified. Besides the widely studied ligands of receptor protein tyrosine kinases, certain agonists of G protein-coupled receptors also are capable of stimulating DNA synthesis in responsive cells. Examples of the latter class of mitogens include the protease thrombin (1, 2) and the lipid lysophosphatidic acid (LPA) (3–5). These otherwise unrelated agonists stimulate DNA synthesis in quiescent fibroblasts through a pertussis toxin (PTX)-sensitive signaling pathway (1–3, 5, 6). This assigns a central role for a heterotrimeric G protein of the G_i subfamily in thrombin- and LPA-induced mitogenesis; however, the nature of the G_i-mediated signal(s) is currently a matter of speculation.

Thrombin cleaves and thereby activates its cognate seven-transmembrane-domain receptor (7) to trigger G protein-dependent stimulation of phospholipase C and PTX-sensitive inhibition of adenylate cyclase (1, 2, 7–10); a synthetic peptide corresponding to the new N terminus of the cleaved receptor can serve as a full agonist of the cloned thrombin receptor (7–10). LPA, a bioactive phospholipid (5) produced and released by activated platelets (T. Eichholtz and W.H.M., unpublished results), appears to bind to its own G protein-coupled receptor to activate effector systems similar to thrombin (3–6, 11, 12). Yet it appears that activation of the phospholipase C–protein kinase C pathway is neither required nor sufficient for LPA- and thrombin-induced DNA synthesis, while it is doubtful whether PTX-sensitive inhibition of adenylate cyclase provides a bona fide mitogenic signal (for review see refs. 2 and 6). This implies that alternative G protein–effector routes must exist to account for mitogenesis.

A major signaling event induced by ligands of receptor tyrosine kinases, such as epidermal growth factor (EGF) or insulin, is the rapid activation of the protooncogene product p21^{ras}, as measured by the transition from its inactive, GDP-bound state to the active, GTP-bound conformation (13–16). Although its function is still a mystery, activation of p21^{ras} is generally considered to be essential for peptide growth factors to stimulate DNA synthesis (17–19). Whether ligands of G protein-coupled receptors, particularly thrombin and LPA, also signal through p21^{ras} is not known. To explore this possibility we determined the activation state of p21^{ras} in serum-deprived fibroblasts before and after treatment with thrombin or LPA, using EGF as a control stimulus. Here we demonstrate that p21^{ras} is rapidly and transiently activated by thrombin receptor agonists and LPA. Thrombin- and LPA-induced, but not EGF-induced, activation of p21^{ras} is abolished by PTX, indicative of regulation by a G_i-like protein. Intriguingly, p21^{ras} activation is not attributable to previously identified PTX-sensitive effector pathways. Instead, pharmacologic evidence suggests that an intermediary protein tyrosine kinase may be involved in p21^{ras} activation. Our results define a signaling pathway in the action of certain G_i protein-coupled receptors, and we suggest that this pathway plays an important role in mitogenesis.

MATERIALS AND METHODS

Materials. Genistein was from Calbiochem; suramin, from Bayer (Wüppertal, F.R.G.); endothelin, from Cambridge Research Biochemicals (Harston, U.K.); and EGF, from Collaborative Research. Human α-thrombin, LPA (1-oleoyl) and other lipids, arachidonic acid, phorbol ester, cholera toxin, and other reagents were from Sigma. PEI-cellulose F plates were from Merck and protein G Sepharose-4 fast flow beads were from Pharmacia. PTX was from List Biological Laboratories (Campbell, CA). [^{32}P]Orthophosphate ($^{32}P_i$) and [^{3}H]thymidine were from Amersham. Ton Maassen (Leiden University) provided polyclonal anti-phosphotyrosine antiserum.

Cell Lines. Rat-1 cells (3) and hamster lung fibroblasts (CCL39, American Type Culture Collection) were routinely grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

DNA Synthesis. Confluent cells in 24-well culture plates were rendered quiescent by incubation in serum-free DMEM for 24 hr. Agonists were then added, and after 8 hr (CCL39

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Abbreviations: LPA, lysophosphatidic acid; PTX, pertussis toxin; EGF, epidermal growth factor.
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cells) or 16 hr (Rat-1 cells) cells were exposed to [^{3}H]thymidine (0.5 μCi/ml; ≈3 μM; 1 Ci = 37 GBq) for an additional 16 hr (CCL39 cells) or 6 hr (Rat-1 cells). Trichloroacetic acid-precipitable material was dissolved in 0.1 M NaOH and quantified by liquid-scintillation counting. The average basal [^{3}H]thymidine incorporation was about 6000 dpm for Rat-1 cells and 13,000 dpm for CCL39 cells (normalized with respect to a 6-hr [^{3}H]thymidine pulse and equal cell density). Agonist-induced relative increases were independent of the presence of unlabeled thymidine (3 μM).

Activation of p21^{ras}. Confluent cells in six-well tissue culture plates were serum-starved for 20 hr in fresh DMEM and then exposed to phosphate-free DMEM supplemented with ^{32}P$_i$ at 200 μCi per well for an additional 4 hr; a ^{32}P-labeling period of 18 hr gave essentially the same results (E.J.V.C., unpublished data). Cells were treated with agonists and incubations were terminated by washing with ice-cold phosphate-buffered saline (PBS). Cells were lysed in a 1% Triton X-114 buffer and p21^{ras} was immunoprecipitated with Y13-259 monoclonal antibody (provided by M. E. Furth, Oncogene Science) and protein G Sepharose and then assayed for bound guanine nucleotides by TLC separation (PEI-cellulose plates) as described (15). After autoradiography, the labeled GTP and GDP spots were scraped off the TLC plates and the relative amounts (corrected for phosphate incorporation) were determined by liquid-scintillation counting.

Protein Tyrosine Phosphorylation. Rat-1 cells were prelabeled with ^{32}P$_i$ (200 μCi/ml) for 4 hr. Cells were lysed in 1 ml of ice-cold lysis buffer (50 mM Tris·HCl, pH 7.5/150 mM NaCl/1% Triton X-100/1% deoxycholate/0.1% SDS/2 mM EDTA/0.06 trypsin inhibitor unit of aprotinin per ml/20 μM leupeptin/1 mM phenylmethylsulfonyl fluoride/500 μM orthovanadate). Lysates were clarified by centrifugation (15,000 × *g* for 10 min at 4°C) and precleared by incubation with 2 μl of normal rabbit serum for 30 min, followed by adsorption onto Formalin-fixed *Staphylococcus aureus* Cowan I bacteria (*Staph. a.*) for 30 min. The *Staph. a.* immune complexes were precipitated and two additional *Staph. a.* incubations (15 min.) and precipitations were carried out. The precleared lysates were incubated with a polyclonal anti-phosphotyrosine antibody for 3 hr at 4°C. Antibody–protein complexes were precipitated with *Staph. a.* for 1 hr on ice. Immunoprecipitates were washed six times with lysis buffer, and bound proteins were eluted by boiling in SDS sample buffer for 5 min followed by SDS/PAGE (5–15% polyacrylamide gradient) and autoradiography.

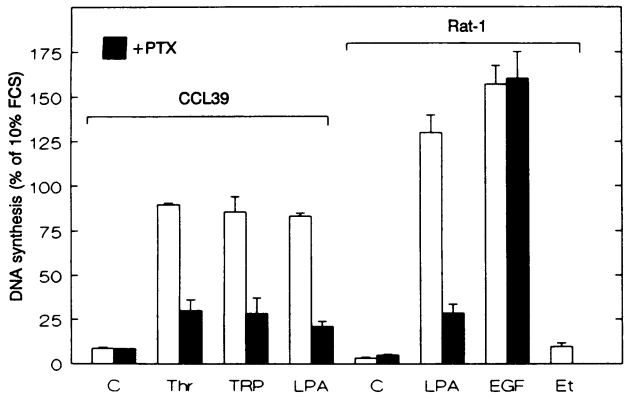

FIG. 1. Agonist-induced DNA synthesis in quiescent fibroblasts. Quiescent, serum-deprived cultures of hamster CCL39 or Rat-1 cells were exposed to thrombin (Thr; 2 units/ml, ≈20 nM), thrombin receptor agonist peptide (TRP; 50 μM; sequence SFLLRNPNDKYEPF; refs. 7–10), 1-oleoyl LPA (100 μM), EGF (10 ng/ml), or endothelin (Et; 100 nM) in the absence or presence of PTX (100 ng/ml) as indicated. C, control without any agonist. Mitogenic stimulation was assayed as described in the text. Data points were normalized with respect to 10% (vol/vol) fetal calf serum (FCS). Values represent mean ± SEM from at least three independent experiments, each performed in triplicate.

RESULTS AND DISCUSSION

Stimulation of DNA Synthesis. Fig. 1 shows that treatment of quiescent CCL39 cells with thrombin, a 14-amino-acid peptide ligand of the cloned thrombin receptor (7–10), or 1-oleoyl LPA results in a significant mitogenic response, which is largely inhibited by PTX. Notably, the peptide ligand does not require the presence of synergizing growth factors to induce DNA synthesis (Fig. 1). The latter result supports the findings of Hung *et. al.* (8) but contrasts to those of others (9), who reported that the peptide agonist fails to mimic thrombin's mitogenic action in CCL39 cells unless an

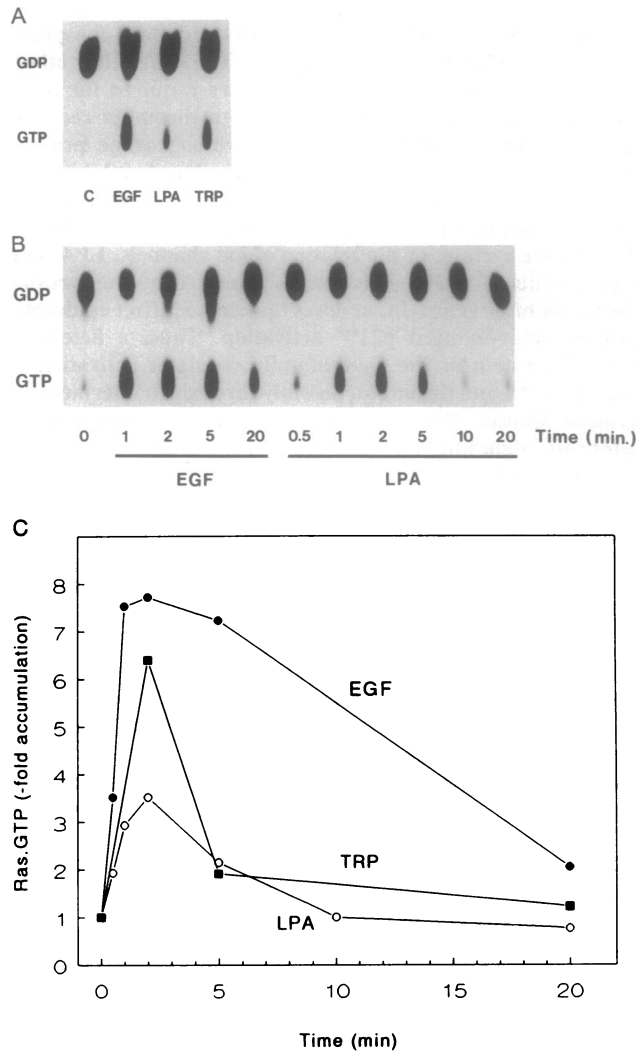

FIG. 2. Activation of p21^{ras} by various agonists. (*A*) Activation of p21^{ras} in CCL39 cells treated for 2 min with EGF (10 ng/ml), 1-oleoyl LPA (1 μM), or thrombin receptor agonist peptide (TRP, 50 μM). Cells were metabolically labeled with ^{32}P$_i$ and p21^{ras} was immunoprecipitated with monoclonal antibody Y13-259; the guanine nucleotides bound to p21^{ras} were then determined as described in the text. (*B*) Time-dependent accumulation of p21^{ras}-GTP in Rat-1 cells treated with EGF (10 ng/ml) or LPA (1 μM). (*C*) Relative amounts of GTP bound to p21^{ras} plotted as a function of time after addition of EGF (10 ng/ml) or LPA (1 μM) to Rat-1 cells and thrombin receptor agonist peptide (TRP, 50 μM) to CCL39 cells. Data points for Rat-1 cells are derived from the experiment in *B*; similar time courses were observed in two other experiments.

additional growth factor is present; the reason for this discrepancy is unclear, but it may reflect subclone variability.

Fig. 1 also shows that Rat-1 cells are highly responsive to both LPA and EGF, with the response to EGF being insensitive to PTX (Fig. 1; cf. ref. 3). The vasoactive peptide endothelin, a potent inducer of phospholipases C and D in Rat-1 cells (12, 20), has little or no effect on DNA synthesis when added alone to these cells (Fig. 1). Unlike thrombin receptor agonists and LPA, endothelin appears to have an absolute requirement for cooperating growth factors such as EGF or insulin to stimulate DNA synthesis (6, 21).

Activation of p21^{ras}. We next examined the above agonists for their ability to activate p21^{ras} in their target cells. In quiescent Rat-1 or hamster CCL39 fibroblasts, endogenous p21^{ras} is almost entirely (92–98%) in the inactive, GDP-bound, form. Fig. 2 *A* and *B*) shows that 1-oleoyl LPA, thrombin receptor agonist peptide, and EGF induce a rapid accumulation of GTP on p21^{ras} in responsive cells. Thrombin (2 units/ml) evoked a response indistinguishable from that to the peptide agonist (not shown). GTP levels are already significantly increased within 30 sec and are maximal at 2 min after agonist addition (Fig. 2 *B* and *C*); thereafter, they gradually return to prestimulation values (Fig. 2*C*). The level of p21^{ras}·GTP rises to ≈20% of total p21^{ras} in response to LPA and to ≈45% with EGF, an increase of about 3-fold and 7-fold above basal, respectively; a 5- to 6-fold increase was induced by the thrombin receptor agonist peptide. The response to LPA is dose-dependent, with an estimated EC_{50} as low as 20–30 nM, a value similar to that for other immediate responses to LPA (11) and in accord with receptor binding data (4); [note, however, that micromolar concentrations are required for long-term mitogenesis (3)]. As expected, LPA-induced p21^{ras} activation is blocked by suramin (0.5 mg/ml), an inhibitor of LPA-receptor binding (4) (Fig. 3). Monoacylglycerol [the major cellular breakdown product of LPA (22)], other lipids, and arachidonic acid have no significant effect on p21^{ras}·GTP levels (Fig. 3).

What receptor-linked effector pathway is responsible for p21^{ras} activation? Although LPA and thrombin receptor agonists trigger phospholipid hydrolysis through activated phospholipases (2, 3, 5–12, 20), the following findings indicate that these effectors cannot account for p21^{ras} activation. First, the peptide endothelin, a much stronger inducer of phospholipid breakdown than LPA in Rat-1 cells (12, 20) without being mitogenic by itself (Fig. 1), fails to mimic LPA in activating p21^{ras} (Fig. 3). Second, the protein kinase C-activating phorbol ester phorbol 12-tetradecanoate 13-acetate (TPA) does not promote p21^{ras}·GTP accumulation (Fig. 3). Last, prolonged treatment (24 hr) of the cells with TPA (100 ng/ml) in an attempt to down-regulate protein kinase C does not attenuate p21^{ras} activation by LPA and thrombin receptor agonists (data not shown).

Thrombin receptor agonists and LPA also trigger opening of ion channels, resulting in immediate membrane depolarization (K. Jalink and W.H.M., unpublished results), a response very similar to that evoked by whole serum (23, 24). However, artificially depolarizing the cells by using a 130 mM KCl/20 mM NaCl incubation medium failed to activate p21^{ras} (not shown), implying that receptor-mediated modulation of ion channels is not responsible for p21^{ras} activation.

Sensitivity to PTX. As shown in Fig. 4, pretreatment of the cells with PTX (100 ng/ml for 3 hr) largely abolishes LPA- and thrombin receptor agonist-induced activation of p21^{ras} without affecting the response to EGF. Thus, the inhibitory effect of PTX on p21^{ras} activation correlates well with that on DNA synthesis (Fig. 1) and would be consistent with p21^{ras} activation being the result of G_i-mediated inhibition of adenylate cyclase. If so, raising cellular cAMP levels should prevent p21^{ras} activation. Contrary to this prediction, however, stimulation of cAMP synthesis by cholera toxin (2 μg/ml; 3-hr pretreatment) or the β-adrenergic receptor agonist isoproterenol (10 μM) does not reduce p21^{ras} activation by either LPA or thrombin receptor agonists (Fig. 4 *Lower* and data not shown). Moreover, the cell-permeant cAMP

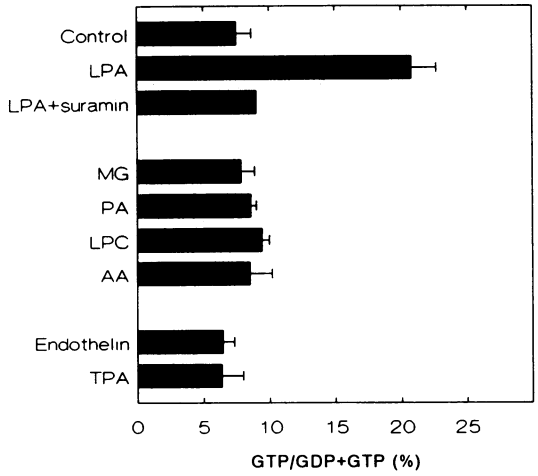

FIG. 3. Analysis of p21^{ras}·GTP accumulation in Rat-1 cells. Cells were treated for 2 min with the indicated agents and the relative amounts of bound GTP were determined. Concentrations used: LPA, 1 μM; suramin, 0.5 mg/ml (10-min pretreatment); 1-oleoylglycerol (MG), 10 μM; 1,2-dioleoyl phosphatidic acid (PA), 10 μM; 1-oleoyl lysophosphatidylcholine (LPC), 10 μM; arachidonic acid (AA), 5 μM; endothelin, 100 nM; and phorbol 12-tetradecanoate 13-acetate (TPA), 200 ng/ml.

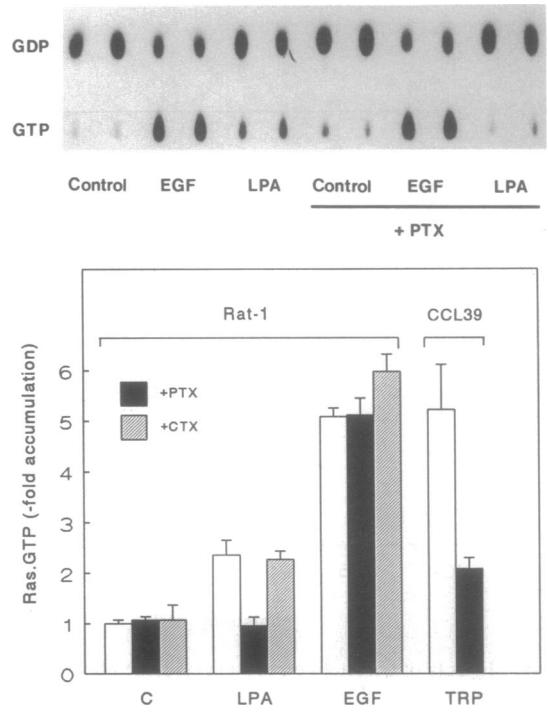

FIG. 4. Effects of bacterial toxins on agonist-induced p21^{ras} activation. (*Upper*) Effect of PTX (100 ng/ml; 3-hr pretreatment) on EGF (10 ng/ml) and LPA (1 μM) -induced p21^{ras} activation in Rat-1 cells. Assays were performed in duplicate as indicated. (*Lower*) Effects of PTX (100 ng/ml) and cholera toxin (CTX, 2 μg/ml) pretreatment (3 hr) on p21^{ras}·GTP accumulation in cells stimulated for 2 min with LPA (1 μM), EGF (10 ng/ml), or thrombin receptor agonist peptide (TRP, 50 μM). C, unstimulated controls. Quiescent ^{32}P-labeled cells were treated with the indicated agents and assayed for p21^{ras} activation. Values represent the mean ± SEM of at least three independent experiments, each performed in duplicate.

analogue 8-Br-cAMP (1 mM, 10-min pretreatment) neither affected basal $p21^{ras}$-GTP levels nor inhibited the response to LPA. Together, these results suggest that a G_i-like protein regulates $p21^{ras}$ activation, while they strongly argue against the possibility that $p21^{ras}$ activation is secondary to a G_i-mediated fall in basal cAMP.

Selective Inhibition by Genistein. Given the central role of $p21^{ras}$ in signal transduction downstream from receptor protein tyrosine kinases (13–17), we sought to correlate LPA- and thrombin-induced $p21^{ras}$ activation with increased tyrosine kinase activity. Like thrombin (25), LPA rapidly stimulates protein tyrosine phosphorylation in responsive fibroblasts (Fig. 5 *Upper*), the details of which will be presented elsewhere (P.L.H., I. Verlaan, and W.H.M., unpublished results). The isoflavone genistein, a protein kinase inhibitor with strong preference for tyrosine-specific kinases (26), inhibits LPA-induced protein tyrosine phosphorylation as well as $p21^{ras}$ activation in Rat-1 cells in a dose-dependent manner ($IC_{50} \approx 10\ \mu M$; Fig. 5 *Lower*). Genistein (50 μM) also inhibits thrombin receptor peptide-induced $p21^{ras}$ activation in CCL39 cells (Fig. 5 *Lower*). Although it should be kept in mind that genistein may have nonspecific side effects, its inhibitory action is remarkably selective in that the drug, at

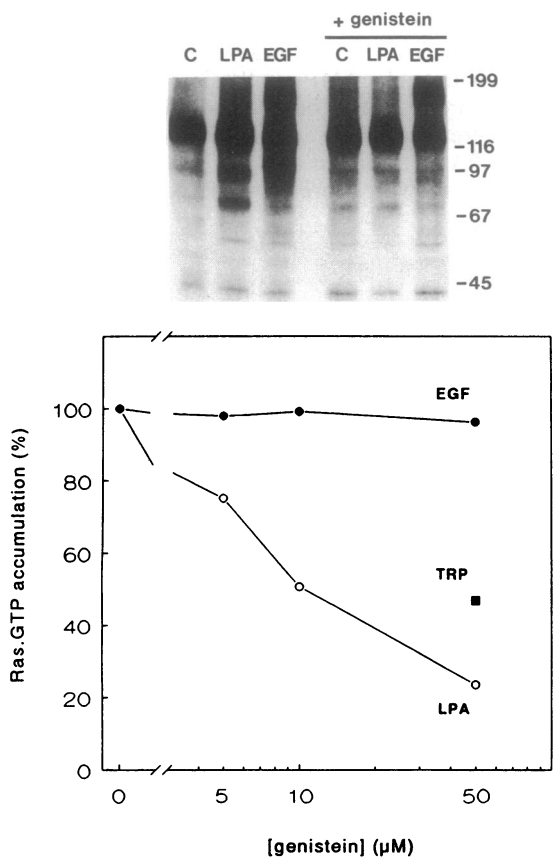

FIG. 5. Effects of genistein on protein tyrosine phosphorylation and $p21^{ras}$ activation. (*Upper*) Protein tyrosine phosphorylation pattern in Rat-1 cells treated for 2 min with LPA (1 μM) or EGF (10 ng/ml) in the presence and absence of genistein (50 μM; 10-min pretreatment). C, unstimulated controls. ^{32}P-labeled proteins were precipitated with anti-phosphotyrosine antibody and analyzed as described in *Protein Tyrosine Phosphorylation*. Note LPA-induced tyrosine phosphorylations of proteins with apparent molecular masses of 120, 90, and 80 kDa, EGF-induced autophosphorylation of the receptor (170–180 kDa), and selective inhibition by genistein. (*Lower*) Dose dependency of genistein (10-min pretreatment) on $p21^{ras}$ activation (values normalized to 100%) in response to EGF (10 ng/ml), LPA (1 μM), or thrombin receptor agonist peptide (TRP, 50 μM) in Rat-1 and CCL39 cells, respectively.

50 μM, has no effect on EGF-induced $p21^{ras}$ activation or on receptor autophosphorylation (Fig. 5). Only at genistein concentrations >100 μM does the response to EGF become significantly inhibited (not shown); this agrees with results in A431 cells, where relatively high concentrations of genistein are required to inhibit EGF action (26). Furthermore, genistein (50 μM) did not affect LPA- and thrombin-induced Ca^{2+} mobilization (K. Jalink, and W.H.M., unpublished results). Taken together, these results suggest that LPA- and thrombin-induced activation of $p21^{ras}$ depends on increased protein tyrosine kinase activity.

Concluding Remarks. We have shown that two distinct agonists of G protein-coupled receptors, LPA and thrombin, bypass known effector pathways to activate $p21^{ras}$ in quiescent fibroblasts, apparently through a PTX-sensitive heterotrimeric G_i protein. At present, we do not know the identity of the G_i subunit(s) involved in $p21^{ras}$ activation. One obvious candidate is $G_{i\alpha 2}$, which can functionally couple to the LPA receptor (27) and whose constitutive activation creates a putative oncogene, by the criterion that it induces neoplastic transformation of Rat-1 cells (28, 29). Another challenge is to identify the G_i-modulated effector enzyme(s) that mediate(s) activation of $p21^{ras}$. On the basis of the results obtained with genistein, together with the fact that various receptor tyrosine kinases signal through $p21^{ras}$ (13–19), we propose that a protein tyrosine kinase lies on the route between G_i and $p21^{ras}$.

Growing evidence suggests that established G protein–effector pathways are not, by themselves, sufficient for driving quiescent cells into DNA synthesis (2, 6). The present results reveal a previously unrecognized pathway in the action of certain G protein-coupled receptors, and they support a model in which G_i-regulated $p21^{ras}$ and its downstream targets act in concert with classic second messenger systems to promote cell proliferation. Obviously, further experiments are required to directly address the biological importance and the regulation of this pathway.

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