

Integrins Regulate the Linkage between Upstream and Downstream Events in G Protein-coupled Receptor Signaling to Mitogen-activated Protein Kinase*

(Received for publication, January 19, 2000)

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Receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) can both activate mitogen-activated protein kinase (MAPK), a critical intermediate in the transduction of proliferative signals. Numerous observations have demonstrated that integrin-mediated cell anchorage can regulate the efficiency of signaling from RTKs to MAPK. Recently, a relationship between integrins and GPCR signaling has also emerged; however, little is understood concerning the mechanisms involved. Here, we investigate integrin regulation of GPCR signaling to MAPK, focusing on the P2Y class of GPCRs that function through activation of phospholipase C β . P2Y receptor signaling to the downstream components mitogen-activated protein kinase kinase and MAPK is highly dependent on integrin-mediated cell anchorage. However, activation of upstream events, including inositol phosphate production and generation of calcium transients, is completely independent of cell anchorage. This indicates that integrins regulate the linkage between upstream and downstream events in this GPCR pathway, just as they do in some aspects of RTK signaling. However, the P2Y pathway does not involve cross-activation of a RTK, nor a role for Shc or c-Raf; thus, it is quite distinct from the classical RTK-Ras-Raf-MAPK cascade. Rather, integrin-modulated P2Y receptor stimulation of MAPK depends on calcium and on the activation of protein kinase C.

Normal cells require both soluble mitogenic factors and anchorage to a solid substratum in order to proceed through the cell cycle, while transformed cells have abrogated the anchorage requirement (1). The molecular basis for anchorage regulation of cell growth is rapidly becoming better understood (2). Recent observations have shown that the integrin family of cell adhesion receptors plays a key role in modulating several aspects of mitogenic signal transduction pathways emanating from RTKs¹ (1–6). Thus, in some systems, integrin-mediated

cell adhesion has been reported to increase the efficiency of RTK activation and autophosphorylation (7–11), whereas, in other cases, integrins have been shown to enhance the coupling between upstream and downstream events in the canonical RTK-Ras-MAP kinase signaling cascade (12, 13). As with RTKs, certain GPCRs can also engage in mitogenic signal transduction. A number of GPCRs, operating through several subfamilies of heterotrimeric G proteins have been shown to activate the MAP kinase cascade; this includes receptors for thrombin, bombesin, bradykinin, eicosanoids, lysophosphatidic acid, and various muscarinic, α -adrenergic, and nucleotide (P2Y) agonists (14–19). Thus, signals from numerous GPCRs ultimately converge on the same downstream MAPK module as signals derived from RTKs.

The underlying mechanisms linking GPCRs to mitogenic signaling are only partially understood, and effectors linked to G proteins can influence the pathway leading to MAPK activation in multiple ways (14, 17). A variety of co-actors have been implicated in GPCR signaling to MAPK; these include Ras, RTKs, Src family kinases, other cytoplasmic tyrosine kinases such as Pyk-2, phosphatidylinositol 3-kinase, and protein kinase C isoenzymes (14, 18, 20). Both Ras-dependent and Ras-independent mechanisms of GPCR mediated activation of MAPK have been described. For receptors coupling through G α_q , one possible Ras-independent mechanism is activation of phospholipase C β leading to IP $_3$ and diacylglycerol production, followed by PKC activation (21–23); it is known that PKC can then activate Raf in a Ras-independent fashion (24). GPCRs can also activate the MAP kinase cascade in a Ras-dependent manner (25); thus, signaling from G $\beta\gamma$ -coupled receptors to MAPK is largely Ras-dependent, and involves $\beta\gamma$ subunits, rather than G α subunits (14, 17, 26, 27). Several protein-tyrosine kinase families have been implicated in mitogenic GPCR signaling. Bruton's tyrosine kinase is directly stimulated by G α_q (28), while members of the Src family have also been implicated in linking certain GPCRs to MAPK (29–31). In some circumstances, a mechanism for signaling from GPCRs to MAPK involves a Src-dependent phosphorylation of the EGF receptor and subsequent activation of Ras (32). The FAK-related kinase CADTK (Pyk-2) has also been implicated in mitogenic GPCR signaling, particularly in neuronal cells (33, 34). Recently there have been some important and novel developments concerning the role of receptor endocytosis in GPCR signaling. Thus, dynamin-mediated endocytosis has been shown to be essential for activation of the MAPK cascade by

* This work was supported by National Institutes of Health Grants GM26165 (to R. L. J.) and HL54889 (to J. L. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[‡] This investigation was performed in partial fulfillment of Ph.D. thesis requirements at the University of North Carolina.

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¹ The abbreviations and trivial names used are: RTK, receptor tyrosine kinase; LPA, lysophosphatidic acid; EGF, epidermal growth factor; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; EC, endothelial cell; BIM, bisindolylmaleimide; Fn, fibronectin; IP $_3$, inositol 1,4,5-triphosphate; PLL, poly-L-lysine; MEK, MAP kinase kinase; MEKK, MEK kinase; MBP, myelin basic protein; FAK, focal adhesion kinase; Gö6983, 3-[1-(3-(amidinothiopropyl)-5-methoxy-1H-

indole-3-yl]4-(1H-indol-3-yl)pyrrolidine-2,5-dione; Ro31-8220, 3-[1-(3-(amidinothio)propyl-1H-indol-3-yl)-3-(1-methyl-1H-indol-3-yl)]maleimide; HUVEC, human umbilical vein endothelial cell; RIPA, radioimmune precipitation buffer; TPA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; GA, geldanamycin; CADTK, calcium-dependent tyrosine kinase.

β_2 -adrenergic receptors (35). This theme has been further developed in showing that β -arrestin recruits Src to agonist-occupied β_2 adrenergic receptors, which then traffic to dynamin-regulated, clathrin-coated pits, and are internalized as a necessary part of the signal transduction process (36). Thus, a number of distinct molecular mechanisms are likely to link GPCRs to mitogenic signaling. It is important to note that signaling pathways are often very context-dependent; for example, the linkages between individual G protein subfamilies and MAPK activation are quite different in fibroblasts and neuronal cells (37). Therefore, it is unlikely that any one mechanism will account for all aspects of signal transduction between GPCRs and mitogenic pathways.

Over the last few years, a number of interesting connections between integrins and GPCRs have emerged. For example, several GPCR agonists including bombesin, gastrin, endothelin, and various muscarinic agents can induce activation and autophosphorylation of FAK (38–40), a cytosolic tyrosine kinase that is also responsive to integrin engagement (41). The FAK response to bombesin and related agents depends on the integrity of the cytoskeleton and requires functional Rho GTPase (39). Activation of FAK via muscarinic acetylcholine receptors can be blocked using RGD peptides that interfere with integrin binding to fibronectin and other extracellular matrix proteins, and it has been suggested that muscarinic signaling activates integrins, which then trigger the tyrosine phosphorylation of FAK (40). Integrin-mediated cell anchorage has also been implicated in GPCR signaling to the MAPK pathway. Thus, two groups have reported an attenuation of GPCR signaling to MAPK in cells held in suspension (13, 42). In addition, a recent report has shown that treatment with RGD peptides, or with the actin de-polymerizing agent cytochalasin D, can block LPA receptor or thrombin receptor activation of MAPK in PC12 cells (43). Interestingly, however, there was little effect of these agents in Rat 1 cells, suggesting that the integrin-GPCR connection may be very context dependent.

Although the existence of a relationship between integrins and GPCR signaling seems clear, there is little mechanistic understanding of how this might take place. Here we have examined the role of integrin-mediated cell anchorage in one particular GPCR pathway that activates MAPK. P2Y receptors, presumably linked to $G_{q/11}$ (15, 44), were stimulated with agonists including ATP and UTP. The activation of upstream events, including IP_3 production and calcium transients, was found to be completely independent of cell anchorage. However, P2Y receptor signaling to downstream events, including activation of MEK and MAPK, was essentially completely dependent on integrin-mediated anchorage to the substratum. Thus, integrins regulate the P2Y GPCR pathway by permitting efficient coupling between upstream and downstream events in the signal transduction cascade.

EXPERIMENTAL PROCEDURES

Materials—ATP and UTP were from Amersham Pharmacia Biotech. LPA and angiotensin II (ATII) were from Sigma. Calyculin A, phorbol 12-myristate 13-acetate (TPA), geldanamycin, and genistein were from Calbiochem (San Diego, CA). Fura-2 AM and BAPTA-AM were from Molecular Probes (Eugene, OR). Bisindolylmaleimide (BIM) was from LC Laboratories, while Ro31-8220 and Gö6983 were from Life Technologies, Inc. Purified recombinant MAPK and MEK were kindly provided by Dr. Lee Graves (University of North Carolina, Chapel Hill, NC). Anti-c-Raf (sc-133) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-MEK1 and Shc antibodies were from Transduction Laboratories (Lexington, KY). Anti-CADTK monoclonal and polyclonal antibodies were kindly provided by Dr. Shelley Earp (University of North Carolina, Chapel Hill, NC). Sources of all other reagents have been reported previously (42).

Cell Culture, Adherence to Fibronectin, and Lysate Preparation—ECV304 cells, a human endothelial-like cell line (45, 46) were main-

tained as described previously (42). Primary cultures of human umbilical vein endothelial cells (HUVECs) (Clonetics, San Diego, CA) were grown and maintained according to supplier's recommendations. Mouse 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. GN4 rat liver epithelial cells were maintained in Richter's improved minimal essential medium. PC12 cells were maintained in Dulbecco's modified Eagle's medium-H and 10% heat-inactivated horse serum + 5% fetal bovine serum. Procedures for harvesting cells, plating cells on adhesive substrata, and preparing cell lysates have been described previously (42).

Immunoprecipitation, Western Blots, and Immune Complex Kinase Assays—Immunoprecipitations were carried out as described previously (42). For kinase assays, all steps, unless indicated otherwise, were performed on ice. Cell lysates were incubated with antibody (MAPK2, c-Raf, or MEK1) typically at 1 μ g of antibody/100 μ g of lysate for 2 h. Protein G-Sepharose beads were added and incubated for an additional 1 h. MAPK immunocomplexes were washed once with RIPA buffer (42) and three times with kinase wash buffer (0.25 M Tris, pH 7.5, 0.1 M NaCl). Raf immunocomplexes were washed twice with RIPA buffer and twice with Raf kinase buffer (25 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 0.1 M NaCl, 1 mM dithiothreitol, and 5 μ M ATP). MEK immunocomplexes were washed two times with RIPA buffer and twice with phosphate-buffered saline. Immunocomplexes were next incubated for 30 min at room temperature in a kinase buffer (10 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μ M ATP, 5 μ Ci of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (3000 Ci/ml) containing 5–10 μ g of myelin basic protein (MBP) as a substrate. Raf kinase activity was measured through a two-coupled enzyme assay combining recombinant MEK (0.5 μ g), and ERK2 (1.25 μ g) in the kinase reaction buffer (47). MEK activity was measured in a coupled kinase assay with the addition of 1.25 μ g of recombinant ERK2 in the kinase assay (48). Reactions were stopped by the addition of hot Laemmli sample buffer and boiling for 5 min. A portion of samples were taken prior to the addition of the kinase reaction mixture and used for Western blotting to show equal loading. Proteins were separated on 15% SDS-polyacrylamide gels, dried, and visualized by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Western analysis using enhanced chemiluminescence (ECL kit, Amersham Pharmacia Biotech) was carried out as described (42).

Inositol Phosphate Accumulation—Flasks (185 cm^2) of subconfluent ECV304 cells were prelabeled with 10 μ Ci/ml ^3H myoinositol for 48 h in inositol- and phenol red-free RPMI (RPMI-free) medium containing 0.1% dialyzed fetal bovine serum. Cells were trypsinized, neutralized, and resuspended in RPMI-free medium containing 2% bovine serum albumin and replated at 100% confluence on 48-well Fn-coated (20 μ g/ml) dishes. For suspension conditions, cells were maintained at 37 $^\circ\text{C}$ on a rotator. Prior to analysis, suspension cells were plated on poly-L-lysine (PLL, 100 μ g/ml)-coated 48 well plates. Agonists were added for 15 min in the presence of 10 mM LiCl. Inositol phosphates were separated, eluted, and quantified as described (49).

Ca^{2+} Measurement—Serum-starved HUVECs were replated on glass coverslips coated with either Fn or PLL at a cell density of approximately 25% of confluence. Cells were loaded with Fura-2 AM (1–2 μ M) for 30–60 min, mounted in a flow-through microscopy chamber, and continuously perfused with Hanks' balanced saline solution. Agonists were added for 30-s pulses. Calcium was measured by a ratio imaging method as described (50). Calcium transients in individual cells were recorded and processed using an InCyt Im2 imaging system (Intracellular Imaging Inc., Cincinnati, OH).

RESULTS

G Protein-coupled Receptor Activation of MAPK Is Dependent on Integrin-mediated Anchorage—As with stimuli that act through receptor tyrosine kinases, certain GPCR agonists can also activate MAPK. Here we have evaluated the role of integrin-mediated cell anchorage in signaling to MAPK from P2Y receptors or from the LPA receptor. Both ECV304 cells and HUVECs display several integrins on their surfaces, including substantial amounts of $\alpha_5\beta_1$, an important receptor for the extracellular matrix protein fibronectin (42). When ECV304 cells were stimulated with EGF, with the P2Y receptor ligand ATP, or with LPA, MAPK was strongly activated in cells that were adherent to substrata coated with fibronectin, but not in suspended cells (Fig. 1A). HUVECs stimulated with EGF, ATP, or UTP also showed a marked integrin-dependent activation of MAPK (Fig. 1B). Thus, cells maintained in suspension, or cells

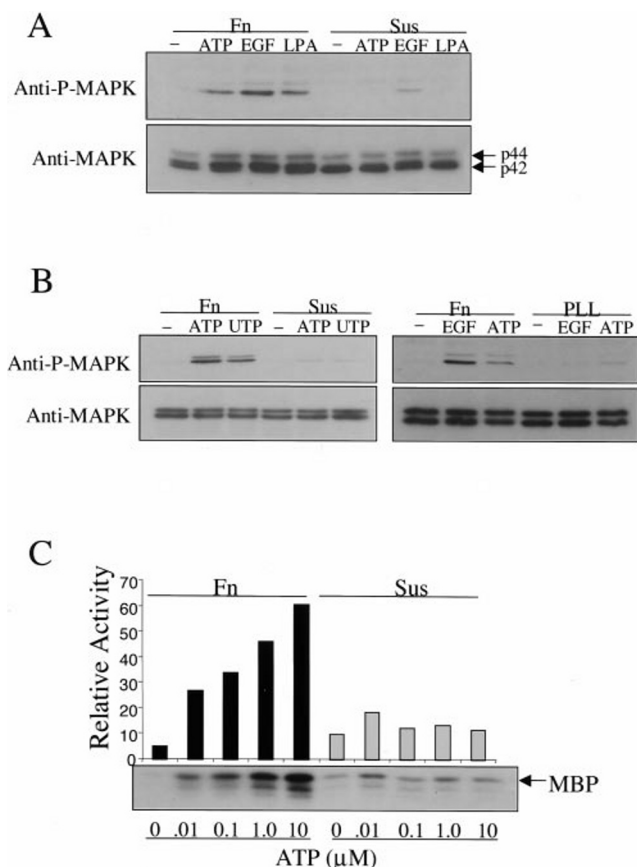


FIG. 1. GPCR activation of MAPK is anchorage-dependent. Confluent, serum-starved ECV304 cells or HUVECs were plated on Fn-coated dishes or PLL-coated dishes, or held in suspension (*Sus*) for 2 h. Where indicated, growth factors (EGF, 10 ng/ml; ATP and UTP, 100 μM; LPA, 10 μM) were added 5 min prior to cell harvest. Cells were lysed and analyzed by Western blotting for MAPK activation. *A*, ECV304 cells; *B*, HUVECs. *Upper panel*, anti-phosphorylated MAPK blot; *lower panel*, anti-MAPK blot to show equal loading. *C*, ATP dose response (0–10 μM ATP) in ECV304 cells. Immunocomplexes were subjected to an *in vitro* kinase assay using [γ - 32 P]ATP and MBP as a substrate. Phosphorylated products were analyzed by SDS-polyacrylamide gel electrophoresis and visualized by PhosphorImager analysis.

nonspecifically adherent to substrata coated with PLL, showed virtually no activation of MAPK, whereas cells adherent via their integrins to fibronectin displayed a prominent activation. Fig. 1C shows the effects of increasing concentrations of ATP on MAPK activation. There was no indication of a dose-responsive activation of MAPK in cells held in suspension, whereas in cells anchored to fibronectin increases in ATP concentration produced a progressive and robust activation of MAPK. In HUVECs and mouse 3T3 fibroblasts, LPA stimulation of MAPK was also found to be dependent on integrin-mediated anchorage (data not shown). The loss of dose-responsive activation of MAPK in cells held in suspension was not due to cell death or irreversible changes; when suspension cells were re-plated on fibronectin, they rapidly regained their ability to respond to agonists by activation of MAPK.²

Thus, in agreement with earlier studies (13, 42, 43), we found that several cell types display anchorage regulation of the ability of GPCRs to signal to MAPK. In further studies we have concentrated primarily on P2Y receptors in ECV304 cells and in HUVECs. In adherent cells, the time course of MAPK activation in response to ATP was similar to that for polypeptide growth factors acting through RTKs; thus, maximal MAPK

activation was seen between 2 and 5 min of agonist treatment, with a gradual return to basal levels by 20–45 min for HUVECs, and 30–60 min for the ECV304 line (data not shown). Our results are consistent with other reports that LPA receptor is coupled to a G_i protein (16) while P2Y receptors are likely coupled to G_{q/11} (44); thus, in ECV304 cells, MAPK activation by LPA was completely blocked by pertussis toxin while ATP-induced activation was unaffected (data not shown).

In summary, as with RTK signaling (9, 12, 42, 51), efficient signal transduction from P2Y and LPA receptors to MAPK requires integrin-mediated cell anchorage. Cells held in suspension culture, as well as cells attached nonspecifically to polycationic surfaces, show marked impairment in the ability of GPCRs to activate MAPK as compared with cells anchored via their integrins.

P2Y Receptor Signaling to MAPK Does Not Recapitulate the RTK Cascade—Recently, there have been several reports implicating transactivation of the EGF receptor or other RTKs in GPCR signaling to MAPK (26, 52–54). This raises the possibility that the anchorage dependence of GPCR signaling to MAPK observed here might simply represent a recapitulation of the previously observed anchorage dependence of RTK signaling to MAPK (12, 13). Here, we evaluated this possibility by examining the activation status of the EGF receptor and its associated adaptor protein Shc. ECV304 cells that were stimulated with EGF showed robust tyrosine phosphorylation of the EGF receptor (Fig. 2A). However, ATP and LPA did not cause any detectable tyrosine phosphorylation of the EGF receptor, and thus do not appear to transactivate this RTK. Fig. 2B shows that, although the EGF receptor was not tyrosine-phosphorylated by ATP or LPA, both of these agonists caused an anchorage-dependent activation of MAPK. Stimulation of the EGF receptor, as well as other RTKs, has previously been shown to increase tyrosine phosphorylation of both the receptor itself and the adaptor protein, Shc (55). We measured Shc tyrosine phosphorylation after stimulation of GPCRs with ATP or LPA. Receptor stimulation with either ATP or LPA did not result in any detectable Shc tyrosine phosphorylation (Fig. 2C) in either anchored or suspended cells. However, in cells that were treated with EGF, we observed an anchorage-independent tyrosine phosphorylation of the 52- and 66-kDa forms of Shc (Fig. 2C). These observations are consistent with the concept that ATP stimulation of P2Y receptors does not transactivate the EGF receptor in the cell types studied here. While we cannot totally rule out the possibility that P2Y stimulation activates a RTK other than EGF receptor, the fact that the ubiquitous adaptor protein Shc is not tyrosine-phosphorylated in response to ATP argues against this possibility. Thus, the anchorage-dependent P2Y receptor signaling to MAPK observed here apparently does not involve transactivation of receptor tyrosine kinases.

Some reports have indicated a role for the calcium-regulated cytoplasmic tyrosine kinase CADTK/Pyk2 in GPCR pathways leading to the activation of MAPK (33, 34). We examined this possibility in the cell types used here. However, we were unable to detect any endogenous CADTK/Pyk2 in either HUVECs or ECV304 cells, although it was readily detectable in PC12 cells and in a hepatocarcinoma line, GN4 (Fig. 2D). Although cells do not show detectable levels of CADTK/Pyk2, they still show expression of FAK, another tyrosine kinase (Fig. 2E). Thus, it seems unlikely that CADTK/Pyk2 would play a role in GPCR signaling in the cells used here. Indeed, there seems little indication of an essential role for tyrosine kinases in P2Y-mediated activation of MAPK in the cell types used in this study. For example, the broad-spectrum tyrosine kinase inhibitor genistein had little effect on ATP-induced activation of

² S. M. Short and R. L. Juliano, unpublished observations.

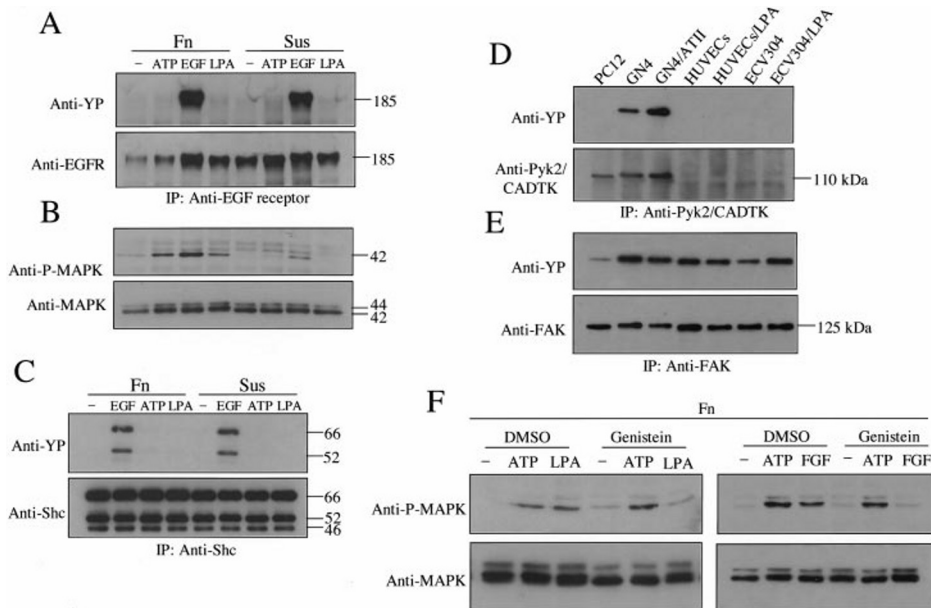


FIG. 2. The role of tyrosine kinases in anchorage-dependent GPCR signaling. In *A–C* and *F*, confluent, serum-starved ECV304 cells were plated on Fn-coated dishes or held in suspension (*Sus*) for 2 h. Where indicated, growth factors (ATP, 100 μ M; EGF, 10 ng/ml; basic fibroblast growth factor, 5 ng/ml; LPA, 10 μ M) were added 5 min prior to cell harvest. Cells were lysed and analyzed by Western analysis for: EGF receptor tyrosine phosphorylation from EGF receptor immunoprecipitates (*A*), MAPK activation (*B*), and Shc tyrosine phosphorylation from Shc immunoprecipitates (*C*). In *D* and *E*, confluent serum-starved PC12 cells, GN4 cells, HUVECs, or ECV304 cells were stimulated with either angiotensin II (*ATII*) or LPA for 5 min where indicated. Cells were lysed and analyzed by Western analysis for Pyk2/CADTK tyrosine phosphorylation from Pyk2 immunoprecipitates (*D*) and FAK tyrosine phosphorylation from FAK immunoprecipitates (*E*). *F*, cells were pretreated with 100 μ M genistein for 2 h and analyzed for MAPK activation in response to ATP, LPA, or fibroblast growth factor. Parallel blots were probed with the appropriate antibodies to show similar loading (*lower panels, A–F*).

MAPK in ECV304 cells (Fig. 2*F*). However, genistein did partially block fibroblast growth factor-induced MAPK activation, a pathway involving a RTK; in addition genistein partially blocked LPA-induced MAPK activation, a pathway thought to involve Src family kinase activity (14). Thus, P2Y receptor signaling to MAPK in these cells does not seem to require a genistein-sensitive tyrosine kinase.

Upstream Events in the P2Y Signaling Pathway Are Anchorage-independent—It is clear that stimulation of P2Y receptors causes activation of MAPK in a manner that is dependent on integrin-mediated anchorage to the substratum, and that this does not involve transactivation of RTKs. It is important then to specify as precisely as possible the locus of anchorage regulation. Thus, we next examined putative pathway components more proximal to heterotrimeric G proteins to further explore the locus of cooperative signaling between integrins and GPCRs. Stimulation of P2Y receptors results in activation of phospholipase C β leading to cleavage of phosphatidylinositol diphosphate and production of diacyl glycerol and IP₃ (21). In response to ATP, we found a dose-dependent accumulation of inositol phosphates that was independent of integrin-mediated anchorage (Fig. 3*A*). Similar results were seen with UTP (data not shown). No inositol phosphate accumulation was observed in response to LPA (data not shown). Both adherent and suspended cells showed similar EC₅₀ values (approximately 1–2 μ M) in response to ATP. Activation of MAPK remained integrin-dependent under experimental conditions where IP₃ production was integrin-independent (that is, including brief binding to a polylysine-coated substratum) (Fig. 3*B*).

Consistent with our inositol phosphate results, the release of intracellular calcium in response to ATP/UTP was independent of integrin-mediated anchorage (Fig. 3, *C* and *D*). LPA did not cause release of intracellular calcium, as anticipated. Fig. 3*D* shows that ATP dose-dependent release of calcium is similar under both adherent and non-adherent conditions. We also used IP₃ accumulation to evaluate the P2Y receptor subtype

activated in the cells used in this study. Our results are in agreement with a recent report that ECV304 cells contain P2Y₂ receptors (56). Thus, both inositol phosphate accumulation and MAPK were activated by ATP and UTP, while we were not able to obtain a response with 2MeSADP, an agonist that is specific for P2Y₁ receptor subtypes (data not shown).

In summary, key events in the P2Y signaling pathway, including generation of inositol phosphate and subsequent calcium release, were found to be independent of cell anchorage. By inference, upstream events including agonist activation of receptor, coupling to G proteins, and activation of the phospholipase C effector must also be anchorage-independent. In contrast, P2Y-mediated activation of MAPK is strongly dependent on integrin-mediated anchorage. Thus, in cells deprived of integrin-mediated anchorage, P2Y signaling events are blocked somewhere between the generation of calcium transients and the activation of MAPK.

Anchorage-dependent ATP-mediated Activation of MAPK Is Regulated by Calcium and by PKC—Although there have been intensive investigations of P2Y mediated signaling to MAPK (34, 57), relatively little work has been done on this issue in the cell types used in this study. Thus, we wished to explore some of the possible steps between the initial activation of phospholipase C β via P2Y stimulation and the accompanying activation of MAPK in endothelial cells. Signaling to MAPK is regulated by calcium in some cell types (58–60). We thus examined whether an increase in cytosolic calcium is involved in the anchorage-dependent activation of MAPK. Fig. 4 (*A* and *B*) shows that, when either ECV304 cells or HUVECs were pretreated with the calcium chelator BAPTA-AM, thus blunting calcium transients, there was a significant attenuation of the ATP-induced MAPK activation. Treatment with the calcium mobilizing agent thapsigargin caused an activation of MAPK, even in the absence of ATP, indicating that an increase in cytosolic calcium *per se* is able to activate MAPK (Fig. 4*C*). However, in thapsigargin-treated cells, there was still a re-

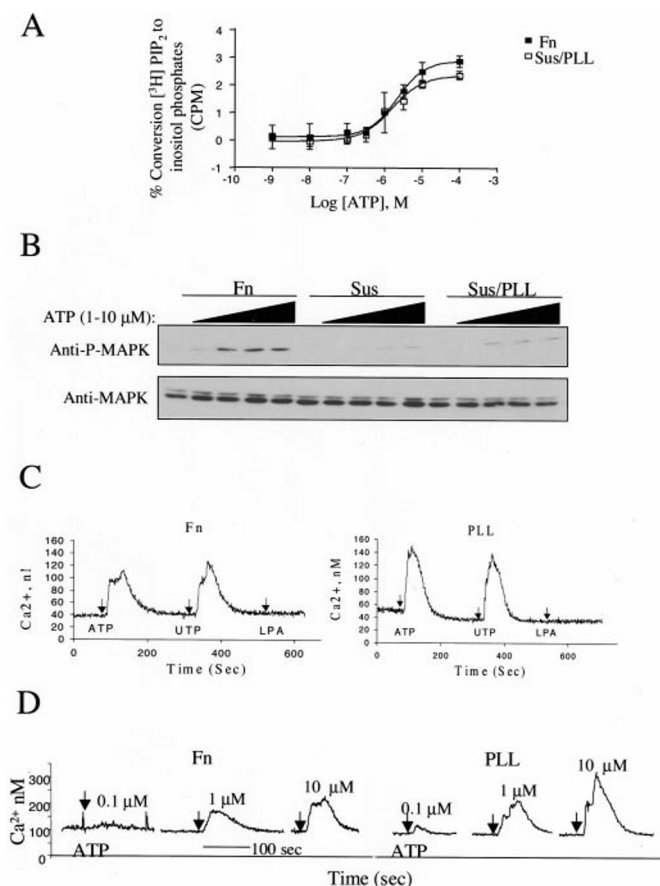


FIG. 3. Events upstream in GPCR signaling are anchorage-independent. A, [^3H]inositol phosphate accumulation was measured in response to 0–100 μM ATP in ECV304 cells plated on Fn for 2 h, or held in suspension (2 h) and then plated briefly on PLL-coated plates (Sus/PLL). All samples were analyzed in triplicate and are represented as the percent conversion of [^3H]phosphatidylinositol diphosphate to inositol phosphates. B, Western analysis of MAPK activation in response to ATP (0–10 μM) under identical conditions used for the inositol phosphate assay. C, integrin-independent release of intracellular calcium in response to 10 μM ATP, UTP, or LPA. Agonists were added for 30-s intervals followed by several minutes of recovery. Data shown are representative for HUVECs and are the average of 8 cells for Fn and 5 cells for PLL. D, concentration-dependent release of intracellular calcium in response to ATP (0–10 μM). Data shown are the average of 2–15 cells for each concentration of ATP.

quirement for integrin-mediated adhesion in order to activate MAPK.

To examine whether a PKC enzyme is involved in P2Y receptor-mediated activation of MAPK, PKC activity was blocked using several common inhibitors, including BIM, Ro31-8220, and G66983 (61), and then the ability of various agonists to activate MAPK was examined. There were minimal effects of the three PKC inhibitors tested on EGF-induced MAPK activation (Fig. 4D). However, the presence of these inhibitors resulted in a substantial reduction in the response of MAPK to ATP, or to the phorbol ester TPA (Fig. 4D). Thus one or more PKC isoforms seem to be involved in P2Y receptor (or TPA) signaling to MAPK, but not in EGF receptor signaling to MAPK. A preliminary evaluation of PKC expression in HUVECs and ECV304 cells by Western blotting indicated that there are 6–7 PKC isoforms expressed in these cell types (data not shown). Because of this complexity, we have not yet been able to identify the specific PKC isoform most closely involved in P2Y receptor activation of MAPK.

The observations described in the preceding paragraphs indicate that both calcium transients and PKC activity contrib-

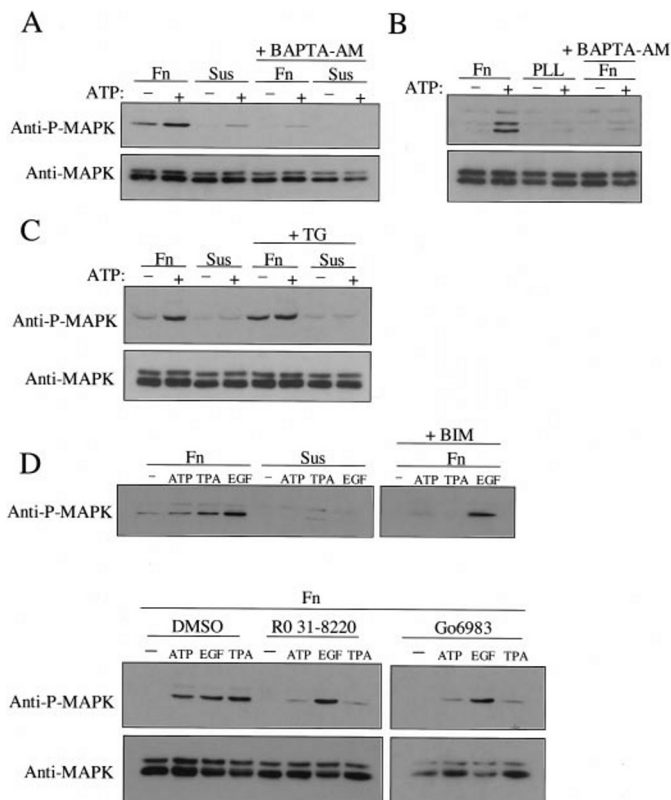


FIG. 4. Anchorage-dependent activation of MAPK by ATP is dependent on intracellular calcium and on PKC. Confluent, serum-starved ECV304 cells or HUVECs were allowed to attach to a fibronectin substratum (Fn) or held in suspension (Sus) or attached to poly-L-lysine (PLL) for 2 h. Where indicated, ATP (100 μM), or EGF (10 ng/ml) were added 5 min prior to cell harvest. TPA (2 μM) was added for 10 min prior to cell harvest. Cells were lysed in a modified RIPA buffer and analyzed by Western analysis for MAPK activation. Where indicated, the calcium chelator, BAPTA-AM (50 μM), was added 5 min prior to the addition of 100 μM ATP (+). A, ECV304 cells; B, HUVECs; C, cells were treated with 2 μM thapsigargin (TG) or with vehicle 10 min prior to the addition of 100 μM ATP (+). For A–C, upper panels, anti-phosphorylated MAPK blot; lower panels, anti-MAPK blot. D, the PKC inhibitors BIM (50 μM), Ro31-8220 (10 μM), or G66983 (5 μM) were added 30 min prior to the addition of ATP, TPA, or EGF; an anti-phosphorylated MAPK blot is shown, as well as loading controls.

ute to P2Y-induced activation of MAPK. However, the precise molecular mechanisms involved are not yet defined.

Evaluation of the Roles of MEK and Raf in P2Y Receptor Signaling to MAPK—We next examined several potential downstream components of the MAPK signaling cascade to further explore possible linkages in the pathway between P2Y receptors and MAPK. First, we measured the activation of MEK. Consequent to ATP or EGF treatment, MEK was strongly activated in cells attached to fibronectin but not in cells held in suspension (Fig. 5A), indicating that MEK, like MAPK, shows a strong anchorage dependence of activation. Next, we evaluated whether Raf was activated in response to ATP. ECV304 cells express abundant levels of c-Raf, very little A-Raf, and no apparent B-Raf (data not shown). As indicated in Fig. 5 (B and C), c-Raf was not activated in response to ATP, while in response to EGF, there was a substantial and statistically significant anchorage-dependent activation of c-Raf, as we have previously reported (12). We also examined the possible role of c-Raf using the pharmacological reagent geldanamycin (GA), which binds to hsp90 proteins and thereby disrupts hsp90-Raf complexes, resulting in a depletion of cytosolic c-Raf (62). Cells were treated with GA and then examined for activation of MAPK. In the presence of GA, there was a marked and statistically significant attenuation of MAPK activity in

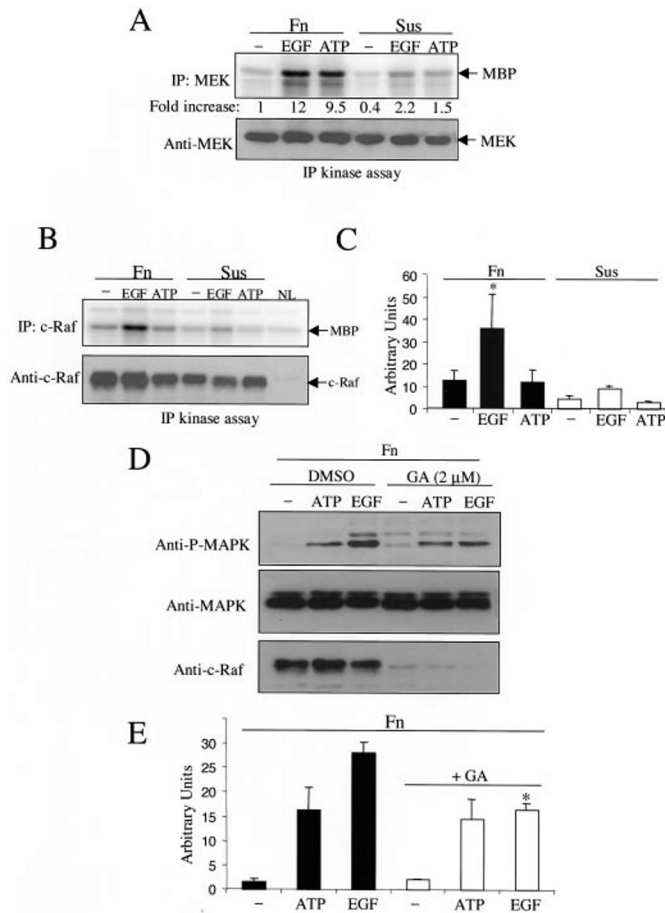


FIG. 5. Analysis of components of the Ras/MAPK cascade to determine the locus of anchorage regulation of GPCR signaling. Confluent, serum-starved ECV304 cells were allowed to attach to a fibronectin substratum (*Fn*) or held in suspension (*Sus*) for 2 h. Cells were stimulated with agonists (EGF, 10 ng/ml; ATP, 100 μM) for 5 min prior to cell harvest. Lysates were analyzed for various kinase activities. **A**, MEK activity was tested by an *in vitro* kinase assay using recombinant ERK2 and MBP as a substrate (*top panel*). The amount of ³²P incorporation into MBP was quantitated using a PhosphorImager. **B**, c-Raf activity was determined by an *in vitro* kinase assay using recombinant ERK2, MEK, and MBP as a substrate (*top panel*). **C**, this chart displays data for c-Raf activation (in arbitrary PhosphorImager units) averaged over four experiments and demonstrates a statistically significant (*, $p < 0.01$) increase in Raf activation in cells stimulated with EGF on Fn, but no significant increase in Raf activation for cells stimulated with ATP (analysis of variance with a Dunnett's post test). **D**, Western analysis of MAPK activation in the presence of the c-Raf inhibitor GA. Where indicated, cells were treated with GA (2 μM) overnight. The *upper panel* shows a Western blot using an anti-active MAPK antibody. The *middle panel* is a loading control for total MAPK. The *lower panel* is a Western blot for total c-Raf protein. **E**, quantitation of bands from Western blots shows MAPK activation (in arbitrary densitometer units) stimulated by EGF or ATP in the presence or absence of GA pretreatment. There is statistically significant (*, $p < 0.01$) reduction of MAPK activation in cells stimulated by EGF but not in cells stimulated by ATP (Student's *t* test). Bands were quantitated using a GS-670 model densitometer (Bio-Rad). Shown is the average and standard deviation from three independent experiments.

cells stimulated with EGF, but there was little or no effect on the activation of MAPK in cells stimulated with ATP (Fig. 5, *D* and *E*). Treatment with GA caused a substantial reduction in the amount of cellular c-Raf but had little effect on levels of MAPK proteins (Fig. 5*E*). Thus, the observed effects of GA on signaling in the MAPK pathway are likely due to depletion of c-Raf. These observations indicate that EGF-triggered stimulation of MAPK involves c-Raf, but the ATP-triggered pathway does not. The observations presented in Fig. 5 (*A–E*) also raise the possibility that MEKKs other than c-Raf (63) may be in-

involved in coupling P2Y receptors to downstream events in the MAPK cascade.

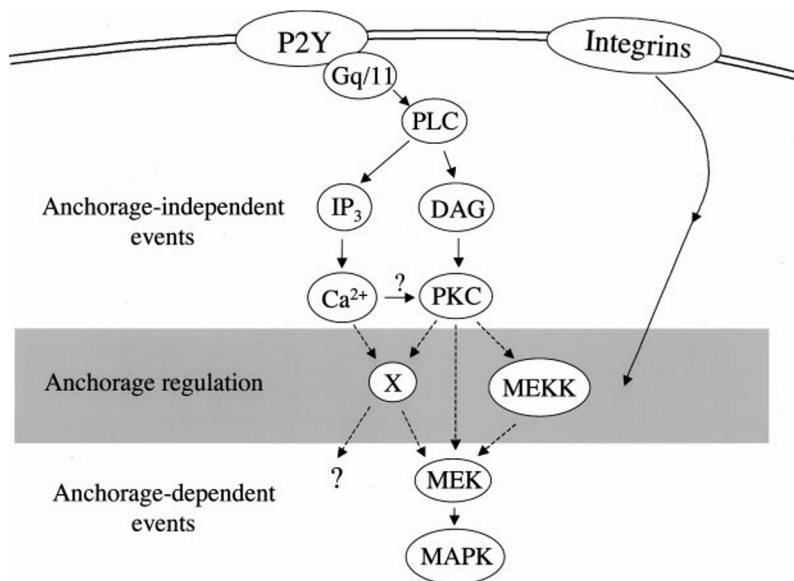
Thus, P2Y receptor stimulation results in anchorage-dependent activation of MEK and MAPK. The role of a MEKK in this process remains undefined since it seems that c-Raf is not activated, and we have not yet identified an alternative mechanism for MEK activation. Therefore, the pathway linking P2Y receptors to MAPK in EVC304 cells and HUVECs seems quite distinct from the canonical RTK-Ras-Raf-MEK-MAPK cascade, but the precise interconnections of the P2Y-MAPK pathway in these cells remain to be fully defined. Regulation of the P2Y-MAPK pathway by integrin-mediated anchorage occurs above the level of MEK activation but below the level of calcium activation.

DISCUSSION

An important new development in signal transduction research is the growing realization that there are critical interconnections between cell adhesion molecules and signaling pathways (5). In particular, the integrin family of cell adhesion receptors has been shown to both directly engage in signaling processes, and to regulate mitogenic signal transduction through RTKs and the canonical Ras-Raf-MAPK cascade (3, 9, 12, 13). In this report, we demonstrate that signals emanating from GPCRs and impinging on MAPK can also be influenced by integrin-mediated cell anchorage, primarily by affecting the linkage between upstream and downstream signaling events. We have focused on the role of anchorage in signaling downstream of P2Y receptor engagement, a pathway that involves the G_q subfamily of G proteins (44); however, the basic phenomenon of anchorage dependence of MAPK activation is also seen for the LPA receptor, which signals through G_i proteins. While many of the experiments reported here have simply compared suspension-cultured cells to cells attached to fibronectin-coated substrata, the regulation of signaling clearly is an integrin-dependent and not merely an attachment-dependent process. Thus, cells attached via nonspecific interactions to a polylysine-coated substratum behave essentially the same as suspension cells, and quite differently from cells attached to fibronectin via integrins. It should be noted, however, that cells display very different morphologies when plated on polylysine *versus* fibronectin. Work from our laboratory and from others has shown that the actin cytoskeleton plays an important role in integrin modulation of signaling by receptor tyrosine kinases (64). This is also likely to be true for GPCR signaling. Indeed, treatment with cytochalasin D sufficient to cause cell rounding strongly inhibits P2Y receptor signaling to MAPK.² However, the precise mechanistic basis for the role of the cytoskeleton is not yet defined.

A variety of GPCRs have been reported to signal to the mitogenic MAPK cascade, and several possible mechanisms have been suggested (14, 18). One plausible mechanism is that GPCRs transactivate the EGF receptor, thus leading to stimulation of the canonical RTK-Ras-MAPK cascade (52). If that were so, then anchorage regulation of GPCR signaling to MAPK might simply be a recapitulation of the previously described anchorage regulation of RTK signaling; however, this does not seem to be the case. We have found no evidence that P2Y agonists activate the EGF receptor or trigger tyrosine phosphorylation of key RTK substrates such as the Shc adaptor protein. In addition, several other aspects of the P2Y signaling process described here, for example the lack of c-Raf activation, suggest that the P2Y pathway is quite different from the classical RTK-Ras-Raf-MAPK cascade delineated for polypeptide growth factor signaling. A very recent report concerning α-adrenergic signaling in PC12 cells also suggests that receptor tyrosine kinases and GPCRs couple to MAPK via distinct path-

FIG. 6. Model for the regulation of MAPK activity by integrins and P2Y receptors in endothelial cells. Stimulation of endogenous P2Y ($G_{q/11}$)-coupled receptors results in anchorage-independent accumulation of inositol phosphates and release of intracellular calcium, and anchorage-dependent activation of both MEK and MAPK. The point of anchorage regulation lies below the release of intracellular calcium and above the activation of MEK. This pathway is independent of tyrosine kinases and Raf and involves PKC. The point of anchorage regulation may involve a MEKK or another component (X), possibly a calcium-activated kinase.



ways (65); this latter report did not deal with the role of cell adhesion, however.

An important consideration here is the locus in the signaling cascade that is regulated by integrin-mediated anchorage. In the case of GPCR signaling, cell anchorage could potentially regulate key upstream aspects of the transduction process including the abundance of cell surface receptors, their coupling to G proteins, or the coupling between G proteins and membrane-bound enzyme effectors such as phospholipase C β . However, this seems not to be the case for anchorage regulation of P2Y receptor signaling. Thus, agonist-induced IP₃ (and presumably diacylglycerol) formation and calcium mobilization take place equally well (or perhaps slightly better) in suspension cells as in cells anchored via integrins. This implies that the entire upstream aspect of the signaling cascade is intact. Our studies have also shown that both calcium mobilization and PKC activation seem to be essential for signal transmission downstream to MAPK. Thus, anchorage regulation of P2Y signaling seems to take place somewhere below the generation of calcium transients and diacylglycerol, but at or above the activation of MEK.

We have examined some possible intermediate components in the P2Y signaling cascade, including Raf and MEK. Clearly, MEK and MAPK act as a module, with both showing dramatic anchorage dependence of activation. As we demonstrate above, it is unlikely that c-Raf has a role in P2Y signaling to MAPK; however, other MEKKs may be able to substitute for Raf (63). In addition, it is clear that a PKC isoform plays an important role, perhaps acting via a MEKK, or by more directly activating MEK and thus MAPK. While many isoforms of PKC can activate MEK, the PKC ζ isoform does this in a c-Raf independent manner (66). It is thus interesting to note that in HEK293 cells, P2Y₂ signaling to MAPK is thought to involve PKC ζ rather than other isoforms (57). However, we have not yet identified the PKC isoform involved in P2Y signaling to MAPK in HUVECs or ECV304 cells. It is also interesting that calcium-mediated activation of MAPK in PC12 cells and epithelial cells has been shown to be independent of Raf (53, 67). In addition to testifying to the complexity of signaling pathways, the above-mentioned examples indicate the existence of calcium-regulated pathways that signal to MAPK in a Ras- and Raf-independent manner. Our current observations on P2Y signaling to MAPK in endothelial-like cells are consistent with a model involving activation of calcium-dependent kinases, and/or of a PKC isoform, leading to activation of the MEK/MAPK module

in a c-Raf-independent manner. A model depicting our current understanding of the P2Y pathway and the possible locus of regulation by integrins is provided in Fig. 6.

The precise mechanism whereby integrin engagement regulates GPCR signaling to MAPK has not yet been clearly defined. A likely possibility involves integrin-mediated recruitment of focal contact and cytoskeletal components that then form a scaffold to allow efficient assembly of the various components of the signaling pathway. For example, both MAPK (9) and some forms of PKC (68) have been reported to be enriched in integrin-dependent focal contacts, thus possibly allowing proximity and efficient interaction of these components. This scaffolding concept has been widely discussed in the context of integrin regulation of RTK signaling (4, 51, 69), as well as in the larger context of regulation of mitogenic signaling cascades (63). In addition, some current models of GPCR function have also emphasized the key role of subcellular localization or compartmentalization in efficient signaling to mitogenic cascades (36, 70). Thus, integrin regulation of GPCR-induced MAPK activation may represent an important example of the relationship between subcellular architecture and the efficient functioning of signal transduction cascades.

Acknowledgments—We extend our thanks to Kevin Laliberte and Arvind Moranham for excellent technical assistance, and Alan Howe, T. K. Harden, and Jesús Mateo for useful scientific discussion.

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