Serine 338 Phosphorylation Is Dispensable for Activation of c-Raf1*

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Numerous extracellular agonists induce consecutive stimulation of Ras guanine nucleotide exchange factors, Ras and c-Raf1, as the starting point of the intracellular mitogen-activated protein kinase cascade. Recent data point to a more complex reaction pattern of this simple sequence. This study was aimed at elucidating the activation process of endogenous c-Raf1 in U937 cells. Treatment of permeabilized U937 cells with the nonhydrolyzable nucleotide guanosine 5′-3-O-(thio)triphosphate (GTPγS) induced prolonged stimulation of Ras and c-Raf1 activity. Intriguingly, both signaling proteins expressed differential responses toward specific inhibitors of phosphoinositide 3-kinases and tyrosine kinases, which indicates diverse signaling reactions feeding into Ras and c-Raf-1. Phosphorylation of c-Raf1 serine 338 by p21-activated kinase has been recently reported to contribute to phosphoinositide 3-kinase-dependent activation of c-Raf1. However, in U937 cells stimulation of c-Raf1 activity by GTPγS did not correlate with p21-activated kinase activity and Ser-338 phosphorylation. Thus Ser-338 phosphorylation appears dispensable for c-Raf1 activation under the conditions used. Together these data deny an essential role for serine 338 phosphorylation in c-Raf1 activation and disclose divergent signaling connections of Ras and c-Raf1 in U937 cells.

The serine/threonine kinase c-Raf1 (hereafter referred to as Raf) is one of the proteins in the cellular MAP1 kinase signal transduction cascade controlling cell proliferation and differentiation among other biological processes. Activation of Raf appears as a multistep process, which is still incompletely understood (for review, see Refs. 1–3). The first step necessary for full activation involves membrane recruitment of Raf via binding to Ras-GTP (4–6). This step is thought to be a prerequisite for subsequent activating events that include phosphorylation on tyrosine residues Tyr-340/Tyr-341 of Raf (7, 8). In addition to Tyr-340/Tyr-341 phosphorylation, several phospho-transfer reactions on serine/threonine residues have been linked to Raf activation (9–11). Protein-protein interactions e.g. with 14-3-3 proteins and heat shock proteins also influence kinase activity (12, 13). Once in its active state, Raf phosphorylates and activates the dual specificity kinase MEK, which in turn facilitates induction of the MAPK ERK.

Class I PI 3-kinases (PI3K) have been shown to express differential effects on the signaling activities of Raf and the other members of the MAPK cascade. In various cell types regulatory effects of PI3K on Ras, Raf, and MEK have been described. At the level of Ras, PI3K have been reported to exert an activating effect, possibly of a conditional nature, on Ras-GTP formation (14–16). On the other hand, PI3K were identified as Ras effectors, binding to and being activated by Ras-GTP (17, 18). Moreover, it was recently proposed that PI3K are involved in phosphorylation of MEK on serine 298 via the Rac/Cdc42 effector PAK. Ser-298 phosphorylation enhances binding of MEK to Raf and can, thus, lead to a sustained activation of MEK (19).

Phosphorylation of distinct serine residues on Raf has been implicated in direct regulation of Raf activity. Two PI3K-sensitive protein kinases affecting Raf kinase activity have been described. Protein kinase B (PKB), which depends on PI3K lipid products for its activation, was recently outlined as a Raf Ser-259 kinase (20, 21). Phosphorylated Ser-259 provides a docking site for 14-3-3 proteins on Raf, and this interaction has been reported to down-regulate Raf activity (22–24). The second published effect is mediated by the serine/threonine kinases PAK2 and -3 (25). PAK is activated via the small GT-Pases Rac and Cdc42 which, at least in some systems, can be regulated by PI3K-dependent activation of guanine nucleotide exchange factors for Rho family GTPases. In this regard, PAK has been shown to phosphorylate Ser-338 in Raf, a phosphorylation site described to be essential for full activation of the kinase (25–27). These findings contrast to other data demonstrating that phosphorylation on Ser-338 does not always correlate with Raf kinase activity (28, 29).

In the present study we have attempted to study the role of PI3K and Ser-338 in activation of endogenous Raf in U937 myelomonocytic cells. A permeabilization strategy has been applied that allows manipulation of the cellular Ras activation independently on PI3K activity status. Using this method we identified a stimulatory effect of PI3K on endogenous Raf, which is not mediated by Ras. Moreover, the system has been exploited to investigate the role of PAK and Ser-338 phosphorylation in the modulation of Raf activity. Our data challenge a role for serine 338 phosphorylation in c-Raf1 activation.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Streptolysin O and GTPγS were purchased from Sigma. For Western blot analysis anti-phospho-ERK (E-10), anti-phosphoserine 473-PKB (Cell Signaling), anti-Raf1, anti-pan-ERK (Transduction Laboratories), anti-PKB (Pharmingen), anti-phosphoserine 338-Raf (Upstate Biotechnologies), anti-pan-Ras clone Ras11 (Calbiochem) were used. βPAK (N-19) and Rac2 (C-21) antibodies were obtained from Santa Cruz Biotechnology. Y13-259 antibody was purified from cell culture supernatant of the corresponding hybridoma cell line (ATCC CRL 1742). Western blots were detected using the enhanced chemiluminescence kit (PerkinElmer Life Sciences). Wortmannin and Genistein were obtained from Alexis Corp., LY297002 was

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1 The abbreviations used are: MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; p21-activated kinase (Akt); PI3K, phosphoinositide 3-kinase; Raf; FAK, p21-activated kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; GTPγS, guanosine 5′-3-O-(thio)triphosphate; MEK RM, His-MEK-K97M; GST, glutathione S-transferase; RBD, Ras binding domain; BSA, bovine serum albumin; TPA, 12-O-tetradecanoylphorbol-13-acetate.
Regulation of c-Raf1 in U937 Cells

from Sigma Chemicals, and the c-Raf inhibitor ZM336372 was purchased from Calbiochem.

Plasmids and Protein Purification—The baculoviruses encoding His-MEK-K97M (MEK KM) and GST-MEK were kind gifts of U. Rapp. Proteins were purified from SF9 cells using nickel nitritotriacetic acid-Sepharose (Qiagen) and glutathione-Sepharose according to standard protocols. Bacterial expression vectors encoding GST-Raf binding domain (RBD) for Raf and GST-PAK RBD were kindly provided by J. Downward and G. Bokoch, respectively. The fusion proteins were expressed in Escherichia coli and purified via affinity chromatography using glutathione-Sepharose (Amersham Biosciences) as described (16).

Cell Culture—U937 cells were cultured in RPMI1640 media supplemented with 10% heat-inactivated fetal calf serum in a humidified atmosphere at 37°C. Before experiments cells were serum-starved for 16–18 h in RPMI media supplemented with 0.1% fatty acid free bovine serum albumin (BSA) and 50 mM HEPES, pH 7.4.

Permeabilization of U937 Cells—Permeabilization was carried out essentially as described by Stephens et al. (30). The bacterial toxin streptolysin O was used to allow influx of GTPyS as well as of larger molecules such as antibodies. Before the experiments the cells were serum-starved for 16–18 h and subsequently washed 3 times with HBBSS (15 mM HEPES, pH 7.4, 140 mM NaCl, 5 mM KCl, 2.8 mM NaHCO3, 1.5 mM CaCl2, 1 mM MgCl2, 0.06 mM MgSO4, 5.6 mM glucose, 0.1% fatty acid free bovine serum albumin (BSA) and 50 mM HEPES, pH 7.4, 107 mM potassium glutamate, 2 mM MgCl2, 1 mM EGTA) at 37°C. Thereafter, cells were resuspended at a density of 10⁶ cells/ml in intracellular buffer and kept on ice for 10 min. To start the experiment, aliquots of 625000 cells/ml, and incubated at 37°C for 8 min with or without inhibitor. Afterward, 1.35 ml of permeabilization buffer (50 mM HEPES, pH 7.2, 67 mM potassium glutamate, 1 mM EGTA, 6 mM MgCl2, 0.6 mM CaCl2, 100 µM ATP) containing 3 units of streptolysin O and, where indicated, the appropriate inhibitor, were added. 750 µl of the cell suspension were removed within the first minute for assays at time point zero. All incubations were carried out at 37°C. Additional aliquots were taken from the suspension 2 and 4 min thereafter. The cells were spun down and lysed immediately. Cleared lysates were subjected to Ras and/or Raf and PAK activity assays.

Ras and Raf Pull-down Assay—To capture activated GTP-bound GT-Pases, GST fusion proteins containing the Ras binding domain of Raf or PAK were used as specific probes for activated Ras and Rac, respectively. The assays were carried out essentially as described with some variations (33, 34). Briefly, U937 cells were lysed in 1 ml Ras pull-down buffer (50 mM Tris/HCl, pH 7.5, 1 mM EGTA, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40) containing 100 µM GDP and 20 µM GTPyS. For Rac assays, cells were lysed in 1 ml of buffer containing 50 mM Tris HCl, pH 7.5, 150 mM NaCl, 2 mM MgCl2, 50 mM NaF, 10% glycerol, 1% Nonidet P-40, 100 µM GDP, and 20 µM unlabeled ATP. The cleared lysates were incubated for 30 min with glutathione-Sepharose, and the precipitates were washed three times with Ras or Rac pull down buffer, respectively. Associated Ras or Rac protein was detected by subsequent SDS-PAGE and Western blotting.

Raf and PAK Kinase Assays—U937 cells were lysed in lysis buffer containing 1% Triton X-100, 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM EGTA. Raf kinase activity was immunoprecipitated incubating the cleared lysates with 0.5 µg of anti-Raf antibody and protein G-Sepharose for 2 h. Immunoprecipitates were washed 3 times with lysis buffer and 2 times with kinase buffer (50 mM Tris/HCl, pH 7.5, 0.1 mM EGTA, 13.3 mM MgCl2, 5% glycerol). The kinase reaction mix contained 2.5 µCi of [γ-32P]ATP, 1 µg of kinase inactive His-MEK substrate, and 15 µl unlabeled ATP in 50 µl of kinase buffer. The kinase reaction was carried out at 30°C for 30 min and stopped by adding Laemmli sample buffer to the reactions. The samples were subjected to a 10% SDS-PAGE gel to separate phosphorylated MEK-K97M from the precipitated kinase. [γ-32P]Phosphate incorporation into the MEK substrate was visualized using a Bio-Rad phosphorimaging system. The PAK kinase assay was carried out essentially as described (19). Immunoprecipitated PAK2/3 was incubated with 5 µg of myelin basic protein as a substrate in 50 µl of kinase buffer containing 20 mM Tris/HCl, pH 8.0, 10 mM MgCl2, 1 mM dithiothreitol, 100 µM ATP, and 1 µCi of [γ-32P]ATP at 30°C for 30 min. Afterward the samples were subjected to a 12.5% SDS-PAGE gel to separate the substrate myelin basic protein from the PAK kinase. Phosphate incorporation into the experiment was visualized using a Bio-Rad phosphorimaging system.

GTPyS

| ZM336372 | 0 | 2 | 4 |
|---|---|---|---|
| WB: α-Raf1 | - | - | - |

FIG. 1. Activation of Ras and Raf in U937 cells is sensitive to wortmannin and genistein. U937 cells were starved for 16 h in RPMI medium containing 0.1% BSA and 25 mM HEPES, pH 7.4. Before the experiment, the cells were washed, resuspended in HBBSS buffer at 10⁶ cells/ml, and incubated at 37°C for 5 min. The cells then were preincubated with 100 nM wortmannin, 100 µM genistein, or Me2SO carrier for 10 min and subsequently stimulated with 10% fetal calf serum, 100 nM TPA, 10 µg/ml insulin, or 100 µM MgATP as indicated. The cells were lysed, and the cleared lysates were used for the Ras-GTP pull-down assay (upper panel). Supernatants of the lysates were used for Raf immunoprecipitation and subsequent kinase assay. Phosphorylation of the Raf substrate MEK KM was analyzed by SDS-page and phosphorimaging (lower panel). WB, Western blot.

WB:

FIG. 2. Suitability of the GTPyS approach for measuring Raf kinase activity in U937 cells. U937 cells were starved overnight and prepared for permeabilization as described under “Experimental Procedures.” Samples were taken within the first minute after the addition of the permeabilization mix, before pore formation started (time = 0). One minute after the addition of the permeabilization mix the time course was started, and samples were taken after an additional 2 and 4 min. The cells were lysed, and a Raf kinase assay was performed. Where indicated 25 µl ZM336372 were added to the kinase reaction to test for specificity of the reaction. Phosphorylation of the Raf substrate MEK KM was analyzed. WB, Western blot.

WB:
slight reduction in active Ras accumulation by wortmannin could be due to the fact that PI3K blockade abrogates basal Ras-GTP levels in U937 cells such that formation of active Ras via GTP\textsubscript{S} uptake adds upon a decreased basal level of Ras-GTP in wortmannin-treated cells (16). Irrespective of these considerations, the data in Fig. 3A demonstrate that GTP\textsubscript{S} perfusion of permeabilized U937 cells can be used to activate Ras in a background of PI3K inhibition, which provides the adequate conditions to investigate the input of PI3K at the level of Raf apart from PI3K effects on Ras activity.

We, thus, proceeded to test the effect of PI3K inhibition on GTP\textsubscript{S}-induced Raf activation. In marked contrast to its effects on Ras activation pattern, wortmannin caused a strong decrease in GTP\textsubscript{S}-stimulated Raf kinase activity (Fig. 3B), indicating that PI3Ks regulate Raf activity via a mechanism independent of its action on Ras. Contrary to the effects of wortmannin, preincubation of the cells with the tyrosine kinase inhibitor genistein, which effectively suppressed Ras-GTP accumulation, only minimally affected Raf activation by GTP\textsubscript{S} (Fig. 3B). However, genistein blocked ERK activation in permeabilized U937 cells subjected to the streptolysin O/GTP\textsubscript{S} protocol (Fig. 3B). From these data we conclude (i) the existence of an additional genistein-sensitive step(s) downstream or parallel to Raf needed for MAPK activation by GTP\textsubscript{S} and (ii) the presence of a Ras-independent, GTP\textsubscript{S}-driven pathway for Raf activation that is active in a background of tyrosine kinase inhibition. Indeed, the Ras-neutralizing antibody Y13-259, which interferes with Ras/effector interactions (31), completely abolished accumulation of active Ras as well as Raf activation in permeabilized GTP\textsubscript{S}-loaded U937 cells (Fig. 4) but was unable to block the GTP\textsubscript{S}-dependent appearance of active Raf in cells pretreated with genistein. The data point to a Y13-259-insensitive G-protein that can induce Raf activation in a back-
but does not block GTPγS-induced Raf activation but does not block GTPγS-induced Raf activation. U937 cells were washed and resuspended in intracellular buffer and incubated at 37 °C for 5 min. Before permeabilization, the cells were incubated with 100 μM wortmannin, 100 μM genistein, or MeSO4 carrier for 10 min as indicated. Where indicated 10 μg of Y13-259 antibody were added to the permeabilization mix. After 1 min 1 nm GTPγS was added to the cells, and the first sample was taken immediately. Additional samples were taken after 2 and 4 min as indicated. The upper panel shows a phosphorimaging scan of the phosphorylated MEK KM substrate. The lower panel shows a α-Raf Western blot (WB) to analyze the amount of immunoprecipitated Raf. The activity of Ras was determined using the Ras pull-down method (middle panel).

The lack of correlation of c-Raf1 and ERK activation observed in genistein-treated U937 cells suggests the existence of alternative pathways linking Ras to ERK activation. One obvious candidate in this regard is B-Raf. Several recent reports make a strong case for B-Raf being the major transducer of Ras signals to ERK (2, 32). Indeed we have detected wortmannin-sensitive activation of endogenous U937 B-Raf in response to TPA, serum, and insulin (data not shown). However, investigation of B-Raf action in our system is rendered arduous by the fact that at least five splicing variants of B-Raf are expressed in U937 cells (data not shown). A detailed study aimed at deciphering the role of B-Raf in Ras-dependent ERK activation is currently under way in our laboratory.

Ser-338 Phosphorylation and Raf Activity Do Not Correlate in Their Sensitivities to Wortmannin and Genistein—PI3Ks have been recently proposed to contribute to full activation of Raf by inducing phosphorylation of Ser-338 in Raf via a Rac/PAK-dependent pathway (27). To test if the requirement for P13K in GTPγS-induced Raf activation in U937 cells correlates with Ser-338 phosphorylation, we performed Western blots using a well characterized phosphoryserine 338-specific antibody (28). Fig. 5 shows that Raf became phosphorylated on Ser-338 upon GTPγS loading of U937 cells. Treatment with wortmannin did not significantly alter GTPγS-dependent Ser-338 phosphorylation, although a slight decrease was observed in some experiments. Moreover, the previously observed Ras-independent activation of Raf by GTPγS in genistein-treated cells was not accompanied by Ser-338 phosphorylation. These data indicate that Ser-338 phosphorylation does not correlate with Raf activity. Moreover, P13K seem to be dispensable for Ser-338 phosphorylation on Raf in U937 cells loaded with GTPγS.

PAK Activation Correlates with Ser-338 Phosphorylation on Raf but Not with Raf Activity—The small GTPase Rac and its effector PAK have been described as mediators of P13K-dependent Ser-338 phosphorylation in other systems. To collect further evidence for the role of this presumptive pathway in U937 cells, we investigated the activation status of both proteins in our experimental setting. Permeabilization of U937 cells in the presence of GTPγS led to a time-dependent activation of PAK (Fig. 5). GTPγS-induced PAK activation was not abolished by preincubation of the cells with wortmannin at the same concentration which efficiently blocked Raf, ERK (Fig. 3B), and PKB activation (data not shown) but was completely blunted by pretreating the cells with genistein (Fig. 5). To complement these results, we also assessed the activation status of the GTPase Rac2 as the upstream activator of PAK. GTPγS-stimulated Rac2 activation showed an analogous activation pattern to PAK, with wortmannin only causing a minor reduction in active Rac2 formation and genistein having a more severe effect. Both Rac2 and PAK activation correlate with Ser-338 phosphorylation, in agreement with the notion that Rac-activated PAK is α the Raf Ser-338 kinase (25). In our hands all three parameters did not correlate with Raf activity induced by GTPγS, indicating that they may not contribute to Raf activation.

Ser-338 Phosphorylation and Raf Activity Do Not Correlate in Space—The results presented above raised the possibility that Ser-338 phosphorylation is not essential for Raf activation in U937 cells. Alternatively, Ser-338 phosphorylation and the unidentified P13K input at the level of Raf may represent two independent sequential or parallel steps, both co-operatively contributing to the Raf activation process. To collect further evidence as to the role of Ser-338 phosphorylation in the Raf activation process, we analyzed the subcellular distribution of Raf after GTPγS loading of permeabilized U937 cells. GTPγS-stimulated U937 cells were lysed in a hypotonic, detergent-free buffer, and cytosolic (S100) and membrane fractions (P100) were separated and tested for Raf activity and Ser-338-phosphorylated Raf (Fig. 6). Although the bulk of Raf protein and Raf activity was present in S100, it was the particulate fraction that exhibited GTPγS and time-dependent appearance of Raf with high specific activity. This is in agreement with the current view on the importance of membrane localization for full Raf activation. In marked contrast, no Ser-338 phosphorylated Raf was detectable in the particulate fraction, whereas a strong signal for phosphoryserine 338 was observed in the soluble fraction. These data show that the majority of Ser-338-phosphorylated Raf did not co-localize with the active kinase. Membrane-bound Ras was used as a marker for the efficiency of the fractionation procedure (Fig. 6) in this experiment. Together these data are in line with the lack of correlation between Ser-338 phosphorylation and Raf kinase activity observed above.

Ser-338 Phosphorylation Does Not Correlate with Raf Activity and MEK Binding Activity of Raf—Next we tried to assess the physical interaction between Raf and MEK after stimulation of intact U937 cells to determine the contribution of Ser-338-phosphorylated Raf to the observed Raf activation. Cell extracts of TPA-stimulated U937 cells were depleted of MEK-
binding proteins by incubation with immobilized GST-MEK or GST as a control, and Raf activity assays were performed on the various fractions (Fig. 7). GST-MEK protein efficiently precipitated Raf kinase activity, whereas GST affinity precipitates contained no detectable MEK-phosphorylating activity. The amount of GST-MEK-bound Raf increased with stimulation in a time-dependent fashion, and the binding decreased after 10 min, correlating with the observed kinetics of Raf stimulation. Whereas the majority of Raf kinase activity was present in pull-down assays produced with GST-MEK, only a minor amount of total Ser-338-phosphorylated Raf was bound to MEK, the bulk of Ser-338 phosphorylation being recovered from the post-GST-MEK pull-down fraction (Fig. 7). This experiment demonstrates that a large portion of Raf protein did not bind MEK and was not activated in response to TPA. The majority of Raf phosphorylated on Ser-338 co-fractionated with inactive Raf, which was unable to bind to MEK, suggesting that Ser-338 phosphorylation is not important for Raf activity and Raf/MEK interaction.

Raf Activity and Ser-338 Phosphorylation Do Not Correlate in Time—To further investigate the relationship between Ser-338 phosphorylation and Raf activation, kinetic experiments have been performed using different agonists to challenge intact U937 cells (Fig. 8). TPA stimulated Raf activation with a peak at 1 min after the addition of the agonist. ERK activation correlated well with the Raf activity under these conditions and was detectable up to 10 min after stimulation. Raf activity decreased faster than ERK activity, indicating that there must be strong negative regulatory events suppressing Raf activity at later time points. Ser-338 phosphorylation on Raf was strongly induced by TPA, but the phosphorylation did not decrease in parallel to the kinase activity. Phosphorylation of Ser-338 was still detectable 30 min after stimulation, a time point at which the Raf kinase activity was back to basal levels (Fig. 8). ATP induced a transient and weaker activation of Raf kinase with a maximum at 1 min. The activity was rapidly down-regulated and no longer detectable 5 min after stimulation (Fig. 8). This property correlates well with the ERK phosphorylation observed in response to ATP. The peak of ERK activation was detected 2 min after stimulation, sharply following the peak of Raf activity. Strong Ser-338 phosphorylation 2 min after stimulation has been observed at a time point when Raf activity was already heavily down-regulated. Similar results were obtained using fetal calf serum and insulin as stimuli (Fig. 8). Therefore, in all cases, regulation of Ser-338 phosphorylation lagged behind Raf kinase activity, providing further evidence against a role of Ser-338 phosphorylation in the Raf activation process.

Ser-338 Phosphorylation Is Dispensable for Hyper Activation of Raf—ZM336372, a potent inhibitor of Raf kinase activity, specifically inhibits Raf kinase activity versus B-Raf and other Ser/Thr kinases in vitro. However, treatment of cells with ZM336372 has been shown to activate Rap, as assayed on immunoprecipitated Raf kinase activity in vitro. It has been postulated that active Rap accumulates in ZM336372-treated cells due to the shutdown of a negative feedback mechanism sparked by Rap itself (33).

We tested the involvement of Ser-338 phosphorylation in Raf
activation after the addition of ZM336372. Incubation of U937 cells with the inhibitor ZM336372 led to the recovery of high MEK-phosphorylating activity in Raf immunoprecipitates (Fig. 9). This activity was much higher than in Raf immunoprecipitates from TPA-stimulated cells (Fig. 9). In line with a previous report (33), ZM336372-mediated activation of Raf was not mirrored by ERK activation in inhibitor-treated cells, suggesting that ZM336372 induced the formation of active Raf while at the same time effectively inhibiting Raf enzymatic activity in the cells. Importantly, ZM336372 did not induce Ser-338 phosphorylation of Raf (Fig. 9), indicating that this phosphorylation event is not a constitutive part of ZM336372-induced processes leading to accumulation of active Raf in U937 cells.

**DISCUSSION**

Ras and numerous other signaling proteins have been shown to affect c-Raf1 activity, but the mechanisms involved in the activation of the kinase in the cellular context are far from being understood. We employed GTP\(\gamma\)S to stimulate endogenous Ras and Raf in permeabilized U937 cells. Activation of Raf by GTP\(\gamma\)S was fully dependent on Ras-GTP formation, as evidenced by the block induced by the Ras-specific neutralizing antibody Y13-259 (Fig. 4). However, the GTP\(\gamma\)S-induced Ras-mediated activation of Raf required the additional input of PI3K since PI3K inhibitors were able to abolish Raf activity under these conditions. Thus, two independent signals from Ras and PI3K appear to be essential for full Raf activation.

Significantly, use of GTP\(\gamma\)S as a stimulus throughout our experiments allowed the exclusion of an effect of PI3K inhibition at the level of Ras because PI3K controls Ras activation in U937 cells via GAP rather than guanine nucleotide exchange factor (GEF) activities (16). PI3K inhibition was reported to blunt basal and agonist-induced Ras activation in intact U937 cells (16). Wennstrom and Downward (34) describe similar effects in COS-7 fibroblast-like cells. The authors demonstrated differential dose-dependencies of Ras-GTP formation and ERK activation toward PI3K inhibition that is indicative of the existence of a PI3K-dependent step between Ras and ERK (34).

In cells stimulated with GTP\(\gamma\)S, treatment with genistein revealed another c-Raf1 activation mechanism independent of Ras. We have previously shown that basal guanine nucleotide exchange by Ras in U937 cells is completely inhibited by high micromolar concentrations of genistein (16). Indeed, GTP\(\gamma\)S did not induce accumulation of active Ras in the genistein background (Fig. 3), but surprisingly, Raf activation was unaltered. Furthermore the Ras-neutralizing antibody Y13-259 did not block GTP\(\gamma\)S-induced Raf activation in the genistein-treated cells, corroborating a radically distinct, Ras-independent Raf activation process. There are few reports in the literature...

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showing Ras-independent activation of Raf, and the underlying mechanisms for these processes are so far poorly defined (35–38). The data imply that G-protein(s) other than a Y13-259-sensitive Ras species can activate Raf in a background of tyrosine kinase inhibition. We have not been able to collect direct evidence as to the nature of the relevant GTPase.

To further investigate the influence of PI3K on Raf activity, we assessed phosphorylation of Ser-338 on Raf, which has been proposed to play an important role in Raf activation (28, 39, 40). Recently, PI3Ks have been implicated as mediators of the Ser-338 phospho-transfer reaction in a pathway involving Rac and its effector PAK in PAK-overexpressing COS7 cells (26, 27). In U937 cells challenged with GTP$^\gamma$/S, Ser-338 phosphorylation can proceed in the presence of PI3K inhibitors. Moreover, TPA induces strong Ser-338 phosphorylation in these cells, although it is well established that TPA does not activate PI3K in U937 cells and related myeloid-derived cells as assessed at the level of phosphatidylinositol 3,4,5-trisphosphate production (30) and PKB phosphorylation (data not shown).

However, GTP$^\gamma$/S induces activation of Rac2, the predominant Rac isoform in leukocytes (41), and PAK to a similar extent in untreated and wortmannin-treated cells, showing that GTP$^\gamma$/S-driven Rac/PAK activation does not rely on PI3K activity. Experimental evidence suggests that PI3K may activate Rac via phosphatidylinositol 3,4,5-trisphosphate-dependent regulation of Rho-family guanine nucleotide exchange factors (42), but also PI3K-independent mechanisms for Rac activation have been reported (43). Our findings show that the accumulation of active Rac in response to GTP$^\gamma$/S is PI3K-independent. The data point to an involvement of tyrosine kinase(s) in the control of Rac activation since genistein treatment severely reduces GTP$^\gamma$/S-driven activation of Rac. The inhibition of Rac correlated well with a block of PAK activation, providing indirect evidence for the existence of a Rac/PAK axis in U937 cells.

GTP$^\gamma$/S-dependent Ser-338 phosphorylation on Raf correlates with Rac and PAK activity under all conditions tested (Fig. 5), providing circumstantial evidence for a causal link between PAK and Raf-Ser-338 phosphorylation in U937 cells. However, PAK activity does not correlate with Raf activity, and in particular, Raf activated as a consequence of Ras-dependent and Ras-independent processes in U937 cells does not exhibit phosphorylation of Ser-338. In line with these findings, Ser-338-phosphorylated Raf is located in the soluble fraction of GTP$^\gamma$/S-challenged U937 cells, whereas Raf with high specific activity accumulates in the particulate fraction and is devoid of

### Fig. 8. Ser-338 phosphorylation on Raf does not correlate with Raf activity in agonist stimulated cells.U937 cells were starved for 16 h in RPMI medium containing 0.1% BSA and 25 mM HEPES, pH 7.4. Before the experiment the cells were washed and resuspended in HBBSS buffer at 10^6 cells/ml. The cells were incubated at 37 °C for 5 min and subsequently stimulated with 100 nM TPA, 100 μM MgATP, 10% fetal calf serum, or 10 μg/ml insulin for the indicated times. The cells were lysed, and the cleared lysates were used for Raf immunoprecipitation and subsequent kinase assay. Phosphorylation of the Raf substrate MEK KM was analyzed by SDS-PAGE and phosphorimaging. Immunoprecipitated kinase was tested for Ser-338 phosphorylation using a Ser-338 phospho-specific antibody (top panel). The cell lysate was used for detection of active ERK (lower panel). Equal loading of lysates was confirmed by probing with a pan-ERK-antibody.

### Fig. 9. The Raf inhibitor ZM336372 induces Ser-338 phosphorylation-independent Raf activity. U937 cells were starved for 16 h in RPMI medium containing 0.1% BSA and 25 mM HEPES, pH 7.4. Before the experiment the cells were washed and resuspended in fresh media at a density of 10^6 cells/ml. Where indicated, 1 μM ZM336372 was added, and the cells were incubated for 1 h. Afterward, the cells were washed 2 times with HBBSS and resuspended in the same buffer at 10^7 cells/ml. The cells were incubated at 37 °C for 5 min and subsequently stimulated with 100 nM TPA or carrier as indicated. The cells were lysed, and the cleared lysates were used for Raf immunoprecipitation and subsequent kinase assay. Phosphorylation of the Raf substrate MEK KM was analyzed by SDS-PAGE and phosphorimaging (top panel). Immunoprecipitated kinase was tested for Ser-338 phosphorylation using a Ser-338 phospho-specific antibody (second panel). The cell lysate was used for detection of active ERK. Equal loading of lysates was confirmed by probing with an ERK antibody. WB, Western blot.
Ser-338 phosphorylation. These findings are in agreement with the study of Chiloeches et al. (44), which reports that in cells overexpressing PAK, Ser-338-phosphorylated Raf was inactive and located in the cytosol (44). In fact, it had been noted earlier also by others that phosphorylation on Ser-338 does not always correlate with Raf kinase activity (28, 29). Furthermore, we find that the majority of Ser-338 phosphorylation does not interact with its downstream substrate MEK and seems to be inactive as a MEK kinase (Fig. 7). Therefore, these data suggest that Ser-338 phosphorylation is not necessary for Raf activation in U937 cells.

Our results point to the existence of several Raf activation mechanisms in U937 cells that do not seem to include Ser-338 phosphorylation. These findings contradict previous work that had made a strong case for Ser-338 phosphorylation as a crucial step in Raf activation (28, 39, 40). All of these studies relied on transient transfection experiments with activated versions of Ras and Src and/or serine 338 to alanine Raf mutants. A caveat inherent to these approaches lies in the prolonged expression of e.g. oncogenic versions of Ras or S338A Raf mutants before the experiment. This may alter the regulatory mechanisms under investigation, e.g. by activation of feedback-mechanisms, affecting upstream regulators of Raf (45–47). Of relevance to the present discussion, a feedback loop negatively regulating Raf kinase activity has emerged in experiments utilizing the novel Raf selective inhibitor ZM336372 (33). Hall-Jackson et al. (33) observe the seemingly paradoxical result that ZM336372 treatment of cells leads to the activation rather than inhibition of Raf as assayed on immunosolated Raf in vitro. In line with these findings, we were able to demonstrate that ZM336372 treatment of U937 cells led to strong activation of Raf in situ as judged by the absence of phosphorylated ERK. Remarkably, ZM336372 did not induce Ser-338 phosphorylation on Raf, indicating that Ser-338 phosphorylation is not required for Raf activity in this feedback mechanism.

In our hands Ser-338 phosphorylation does not correlate with Raf activation under all the conditions described above. Nevertheless these findings cannot exclude that a punctual phosphorylation of Ser-338 might be needed transiently as an initial step in the activation of Raf kinase after Ras binding, e.g. for recruiting binding partners or facilitating a conformational change necessary for further steps in the activation process. However, in our experiments the majority of Ser-338-phosphorylated Raf is inactive, whereas Raf activated under different conditions lacks Ser-338 phosphorylation.

In conclusion, our data demonstrate the existence of a PI3K-dependent step in Raf activation that is distinct from Ras-GTP or Rac-GTP formation and Ser-338 phosphorylation. Although many molecular details about the mechanisms of MAPK regulation by PI3K are emerging, the general mode of the regulatory function of these enzymes is still under discussion (34, 48). The contradictory data in the literature might indicate that the phosphorylation event as well as the activation of Raf in general are regulated in a complex cell type and/or stimulus-dependent manner. Further efforts will be necessary to clarify the pathways from PI3K leading to Raf and the specific function of Ser-338 phosphorylation in the activation process of Raf.

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