Activation of phospholipase Cγ by PI 3-kinase-induced PH domain-mediated membrane targeting

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Signaling via growth factor receptors frequently results in the concomitant activation of phospholipase Cγ (PLCγ) and phosphatidylinositol (PI) 3-kinase. While it is well established that tyrosine phosphorylation of PLCγ is necessary for its activation, we show here that PLCγ is regulated additionally by the lipid products of PI 3-kinase. We demonstrate that the pleckstrin homology (PH) domain of PLCγ binds to phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P3], and is targeted to the membrane in response to growth factor stimulation, while a mutated version of this PH domain that does not bind PtdIns(3,4,5)P3 is not membrane targeted. Consistent with these observations, activation of PI 3-kinase causes PLCγ PH domain-mediated membrane targeting and PLCγ activation. By contrast, either the inhibition of PI 3-kinase by overexpression of a dominant-negative mutant or the prevention of PLCγ membrane targeting by overexpression of the PLCγ PH domain prevents growth factor-induced PLCγ activation. These experiments reveal a novel mechanism for cross-talk and mutual regulation of activity between two enzymes that participate in the control of phosphoinositide metabolism.

Keywords: PH domains/phosphoinositides/phospholipase Cγ/PI 3-kinase

Introduction

Phosphoinositide metabolism plays an important role in the control of diverse cellular processes such as cell proliferation and differentiation, cell survival, and control of cell shape and cell migration (Divecha and Irvine, 1995; Carpenter and Cantley, 1996). A large variety of extracellular signals stimulate the hydrolysis of phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P2] by activation of phospholipase C (PLC) isoforms (Cockcroft and Thomas, 1992; Lee and Rhee, 1995). It is now well established that the two products of this reaction, the intracellular second messengers inositol 1,4,5-trisphosphate [Ins(1,4,5)P3] and diacylglycerol, mediate the release of Ca2+ from intracellular stores and activation of protein kinase C (PKC), respectively (Berridge, 1993; Nishizuka, 1995). The critical role of these pathways in mammalian growth and development is underscored by the recent finding that targeted deletion of PLCγ1 causes embryonic lethality in mice (Ji et al., 1997).

The PLCγ1 and PLCγ2 isoforms are activated by growth factor stimulation of both receptor and non-receptor protein tyrosine kinases (Cockcroft and Thomas, 1992; Rhee and Bae, 1997). Structurally, each PLCγ isoform has a putative pleckstrin homology (PH) domain at its amino-terminus, followed by the two conserved parts of the catalytic domain (themselves separated by two tandem SH2 domains), an SH3 domain and a putative split PH domain. Upon growth factor stimulation, the SH2 domains of PLCγ bind to tyrosine autophosphorylation sites in growth factor receptors, leading to tyrosine phosphorylation and stimulation of PLCγ enzymatic activity. It has been demonstrated that phosphorylation of Tyr783 of PLCγ is essential for stimulation of enzymatic activity in NIH-3T3 cells stimulated with platelet-derived growth factor (PDGF) (Kim et al., 1991). Yet, in many cases, the tyrosine phosphorylation of PLCγ does not correlate with Ins(1,4,5)P3 production; certain extracellular signals lead to weak tyrosine phosphorylation of PLCγ and extensive production of Ins(1,4,5)P3, while other signals cause strong tyrosine phosphorylation of PLCγ but only low levels of Ins(1,4,5)P3 production (Rhee and Bae, 1997). Therefore, additional mechanisms, independent of PLCγ tyrosine phosphorylation, are likely to play an important role in the control of PLC enzymatic activity in response to extracellular stimuli.

There is now good evidence that PH domains of signaling molecules are involved in their regulated targeting to cell membranes (Lemmon et al., 1995, 1996). PH domains are protein modules of ~120 amino acids that have been identified in >100 different proteins (Haslam et al., 1993; Mayer et al., 1993; Musacchio et al., 1993; Gibson et al., 1994). These include protein kinases (Akt/ PKB/Rac, Btk and the β-adrenergic receptor kinase, βARK), guanine nucleotide exchange factors for small GTPases (e.g. DbI and Sos), PLC isoforms (β, γ and δ) and GT-Pase-activating proteins (e.g. Ras-GAP). All PH domain-containing proteins appear to have a functional requirement for membrane association (Gibson et al., 1994), and several recent studies indicate that PH domains play a role in directing this membrane targeting (Cifuentes et al., 1993; Davis and Bennett, 1994; Lombardo et al., 1994; Paterson et al., 1995; Wang et al., 1996; Zheng et al., 1996; Chen et al., 1997; Lemmon et al., 1997).

Efforts to identify the PH domain ligands responsible for membrane binding have led interest toward both protein–
Membrane targeting of PLCγ1 PH domain

Several possible PH domain targets have been proposed for different PH domains, including the βγ subunits (Gβγ) of heterotrimeric G proteins (Touhara et al., 1994; Tsukuda et al., 1994), PKC (Konishi et al., 1994; Yao et al., 1994) and phosphoinositides (Harlan et al., 1994; Garcia et al., 1995; Hyvönen et al., 1995; Lemmon et al., 1995; Pitcher

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**Fig. 1.** (a) Fluorescence micrographs of cells expressing GST–PLCγ1-PH (A–C) or GFP–PLCγ1-PH (D–F) fusion proteins. Confocal immunofluorescence microscopy of IMR33 cells microinjected with GST alone (A) or GST–PLCγ1-PH (B and C) and grown in the absence of serum. Cells were unstimulated (B) or stimulated with fetal bovine serum (C). A diffuse cytoplasmic staining can be seen in starved cells (B). In stimulated cells (C), a distinct accumulation of fluorescence can be seen in the lamellar region of the cell (arrows). Confocal fluorescence micrograph of L6 cells expressing GFP alone (D) or GFP–PLCγ1-PH fusion protein (E and F). In starved cells (E), a diffuse cytoplasmic fluorescence is seen. In serum-stimulated cells (F), a distinct accumulation of fluorescence is seen in the lamellar region of the cell (arrows).

(b) Fluorescence micrographs of L6 cells expressing GFP–PLCγ1-PH at the indicated time points following PDGF stimulation. Bar, 10 μm.
Here we describe studies of the role of the putative PLC\(\gamma\) signaling. PLC\(\gamma\) biological activity of proteins involved in intracellular defined, it is clear that PH domains are important for the plasma membrane (Paterson et al., 1995). Although their precise roles and ligands are not well defined, it is clear that PH domains are important for the biological activity of proteins involved in intracellular signaling. PLC\(\delta\) and Akt/PKB/Rac provide two examples, and a third is provided by the Btk PH domain, in which its substrate PtdIns(4,5)P\(_2\) thus allowing processive PtdIns(4,5)P\(_2\) hydrolysis (Cifuentes et al., 1994). PLC\(\gamma\)1 is targeted to the plasma membrane, in response to serum and growth factor stimulation. More specifically, we find that PLC\(\gamma\)1-PH binds to PtdIns(3,4,5)P\(_3\) and that phosphatidylinositol (PI) 3-kinase activity plays an important role in PLC\(\gamma\) activation and membrane localization in multiple cell types.

### Results and discussion

In order to explore the possible role of the PH domain in signal-dependent localization of PLC\(\gamma\)1, a glutathione-S-transferase (GST) fusion protein containing the PLC\(\gamma\)1 PH domain was microinjected into serum-starved IMR33 cells. The localization of the protein was analyzed using confocal fluorescence microscopy in fixed cells incubated with polyclonal anti-GST antibodies followed by rhodamine-conjugated anti-goat antibodies. As shown in Figure 1aB, the fusion protein is homogeneously dispersed in the cell cytoplasm in the absence of serum stimulation. However, within 5 min of stimulation with either serum (Figure 1aC) or PDGF (Table I), the PH domain was recruited to the plasma membrane. No membrane recruitment was seen when GST alone was used (Figure 1aA). These data

### Table 1. Conditions affecting cellular distribution of PLC\(\gamma\)-PH mutants or isoforms

<table>
<thead>
<tr>
<th>PH domain</th>
<th>Treatment</th>
<th>Cell Intracellular</th>
</tr>
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<tbody>
<tr>
<td>PLC(\gamma)1</td>
<td>untreated</td>
<td>–</td>
</tr>
<tr>
<td>PLC(\gamma)1</td>
<td>serum or PDGF</td>
<td>+</td>
</tr>
<tr>
<td>PLC(\gamma)6/7</td>
<td>serum or PDGF</td>
<td>+</td>
</tr>
<tr>
<td>PLC(\gamma)3/4</td>
<td>serum or PDGF</td>
<td>–</td>
</tr>
<tr>
<td>PLC(\delta)</td>
<td>untreated, serum, PDGF</td>
<td>+</td>
</tr>
<tr>
<td>PLC(\gamma)1</td>
<td>PDGF + Wortmannin</td>
<td>–</td>
</tr>
<tr>
<td>PLC(\gamma)1</td>
<td>PDGF + DN PI 3-kinase</td>
<td>+</td>
</tr>
<tr>
<td>PLC(\gamma)1</td>
<td>activated PI 3-kinase</td>
<td>+</td>
</tr>
<tr>
<td>PLC(\gamma)1</td>
<td>inactive PI 3-kinase</td>
<td>–</td>
</tr>
</tbody>
</table>

The table summarizes the cellular distribution of various PH domain isoforms or mutants following a variety of cellular treatments. Experiments using serum, PDGF or Wortmannin (the top six lines of the table) were done in L6, COS and IMR33 cells. Experiments utilizing dominant-negative, constitutively active or kinase-inactive forms of PI 3-kinase were done in COS cells and HeLa cells.

Although their precise roles and ligands are not well defined, it is clear that PH domains are important for the biological activity of proteins involved in intracellular signaling. PLC\(\delta\) and Akt/PKB/Rac provide two examples, and a third is provided by the Btk PH domain, in which its substrate PtdIns(4,5)P\(_2\) thus allowing processive PtdIns(4,5)P\(_2\) hydrolysis (Cifuentes et al., 1994). PLC\(\gamma\)1 is targeted to the plasma membrane, in response to serum and growth factor stimulation. More specifically, we find that PLC\(\gamma\)1-PH binds to PtdIns(3,4,5)P\(_3\) and that phosphatidylinositol (PI) 3-kinase activity plays an important role in PLC\(\gamma\) activation and membrane localization in multiple cell types.

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Membrane targeting of PLCγ1 PH domain

Fig. 3. (A) Primary structures of PLCγ1-PH mutants. Mutations in the 3/4 and 6/7 loops are shaded. (B) Specificity of [32P]gPI(3,4,5)P3 binding to GST PLCγ1-PH compared with other GST–PH fusion proteins.

show that plasma membrane localization of PLCγ1-PH occurs only in stimulated cells.

The dynamics of PLCγ1-PH localization to the plasma membrane were investigated using the PLCγ1-PH fused to green fluorescent protein (GFP) and transiently expressed in IMR33 fibroblasts, L6 myoblasts, HeLa and COS-1 cells. Due to its intrinsic fluorescence, the subcellular localization and dynamics of the GFP fusion protein can be observed in living cells without the fixing and staining procedures required for indirect immunofluorescence of, for example, GST fusion proteins. Thus, alterations in GFP fusion protein localization resulting from different cellular treatments can be monitored directly, allowing analysis of the dynamic localization events occurring in living cells. Cells were transfected with a vector directing the expression of a GFP–PLCγ1-PH fusion protein. As with the microinjection studies described above, GFP–PLCγ1-PH was found to be present only in the cytoplasm when cells were starved of serum following transfection (Figure 1aE). However, when cells were stimulated with serum (Figure 1aF) or PDGF (Table I), the GFP–PH fusion protein was seen to associate with the plasma membrane within 3–5 min. In control cells expressing free GFP, an intense cytoplasmic fluorescence was seen especially around the nucleus, but no membrane localization was observed in either starved or unstimulated cells (Figure 1aD).

Use of the GFP fusion protein allowed us to follow the time course of PLCγ1-PH membrane association in living cells. Membrane localization was first seen after 3–5 min, and became pronounced at 10 min, appearing as a distinct, rim-like fluorescence along the leading edge of the cell. During the subsequent 10–15 min, highly dynamic changes in this staining pattern were seen; it first became associated with ruffle-like structures, typically located at some distance from the growth substratum. Distinct fluorescent lines of the ruffles, probably representing upward-bent membrane veils, underwent rapid changes in their orientation such that 90° turns could be seen within 1 min (Figure 1b). Extensive membrane ruffling was apparent at 27 min, while from 30 min onwards there was a gradual disappearance of the distinct membrane-associated staining. Thus, PLCγ1-PH recruitment to the plasma membrane in these cells is transient, dependent upon growth factor...
stimulation, and is seen especially in the highly dynamic membrane areas, correlating with the enzymatic activity of PLCγ after growth factor stimulation.

Specific binding of the PLCγ-PH to PtdIns(3,4,5)P₃

Previous studies have demonstrated specificity of phospholipid binding by PH domains. For example, the PH domain of PLCδ₁ binds with high affinity and specificity to PtdIns(4,5)P₂ (Ferguson et al., 1995; Lemmon et al., 1995) while the PH domain of GRP1 binds to PtdIns(3,4,5)P₃ and Akt/PKB binds to PtdIns(3,4)P₂ (Franke et al., 1997; Frech et al., 1997; Klarlund et al., 1997). Binding of PtdIns(3,4,5)P₃ to PH domains has been reported for other proteins containing PH domains, such as Btk (Salim et al., 1996; Kojima et al., 1997) and Akt-1 (James et al., 1996).

To characterize the lipid-binding properties of the PLCγ-PH, we incubated deacylated lipid extracts from PDGF-stimulated [³H]inositol-labeled IMR33 fibroblasts with beads to which the GST–PLCγ-PH was attached. The associated glycerophosphoinositides (gPis) were eluted and analyzed by HPLC. This is a rigorous test of lipid binding, since the ratio of lipids provided in the extract is physiological. The experiment presented in Figure 2A shows that the PH domain of PLCγ₁ binds strongly to PtdIns(3,4,5)P₃. Only small quantities of PtdIns(3,4)P₂ and PtdIns(3)P were bound. The binding of deacylated PtdIns(3,4,5)P₃ was displaced by the water-soluble head group Ins(1,3,4,5)P₄ at a half-maximal concentration of 1–2 μM (Figure 2B). The binding affinity appears to similar to that of the GRP1 PH domain, where the concentration of Ins(1,3,4,5)P₄ that inhibited binding of PtdIns(3,4,5)P₃ half maximally was 3–8 μM (Klarlund et al., 1997).

Binding of wild-type and mutants PLCγ-PH to plasma membrane and to PtdIns(3,4,5)P₃

To analyze further the specificity of the PLCγ₁-PH membrane localization and binding to PtdIns(3,4,5)P₃, and in
order to determine which portion of the PH domain is important for ligand and membrane binding, we analyzed the binding properties of several PH domain mutants. The mutations are reported schematically in Figure 3A. From structural analysis of PLCδ1 PH domain binding to Ins(1,4,5)P3 and PtdIns(4,5)P2, it has been shown that three loops separating β-strands in PH domains (the β1/β2, β3/β4 and β6/β7 loops) are particularly variable in both length and sequence, and are critical for ligand binding. Ins(1,4,5)P3 contacts only these loops in its high-affinity complex with the PLCδ1 PH domain (Ferguson et al., 1995; Lemmon et al., 1995). We individually replaced two of the variable loops of the PLCγ1-PH with the equivalent loops of the dynamin-1 PH domain, which does not localize to the membrane in our studies (data not shown). Figure 3B compares PtdIns(3,4,5)P3 binding to mutated PLCγ1-PH with that to the wild-type domain. Mutation of the β6/β7 loop had no effect upon ligand binding of PLCγ1-PH, while replacement of the β3/β4 loop with the aligned dynamin-1 PH domain sequences completely abolished ligand binding. In agreement with this result, mutation of the β6/β7 loop had no effect on membrane localization, while mutation of the β3/β4 loop abolished membrane localization of the PLCγ1 PH domain (Table I). These results indicate that PLCγ1-PH binding to PtdIns(3,4,5)P3 and to the plasma membrane is sequence specific, and further implicate the β3/β4 loop in key interactions, as seen for Ins(1,4,5)P3 binding to PLCδ1.

**Effect of Wortmannin and dominant-negative PI-3 kinase on PLCγ-PH plasma membrane localization**

The experiments presented above indicate that PLCγ interacts via its PH domain with PtdIns(3,4,5)P3, the major lipid product of activated PI 3-kinase (Traynor-Kaplan et al., 1988; Auger et al., 1989; Stephens et al., 1991). Therefore, several approaches were taken to investigate the role of PI 3-kinase in the translocation and activation of PLCγ. We first tested the effect of PI 3-kinase inhibition on the cellular distribution of the PLCγ-PH using a dominant-interfering mutant of p85 (the regulatory subunit of PI 3-kinase) and the PI 3-kinase inhibitor Wortmannin. The experiments presented in Figure 4 and Table I show that either expression of dominant-interfering p85 or treatment with Wortmannin blocked serum- and PDGF-induced membrane localization of the PLCγ-PH, further indicating that the lipid products of PI 3-kinase serve as targets of the PLCγ-PH in living cells.

**Activated PI 3-kinase-induced PLCγ-PH plasma membrane localization**

Next we examined the effect of expression of a constitutively activated PI 3-kinase on the cellular localization of the GFP–PLCγ1-PH. Expression of membrane-targeted p110β leads to accumulation of PtdIns(3,4,5)P3 and PtdIns(3,4)P2 (Logan et al., 1997). In cells that overexpress this constitutively active PI 3-kinase mutant, the PH domain of PLCγ1 is targeted to the plasma membrane even in serum-starved cells (Figure 4D and Table I). By contrast, membrane localization of the PLCγ PH domain is not seen in cells that express a membrane-targeted, kinase-inactivated form of PI 3-kinase (Table I), providing additional evidence that accumulation of 3’ phosphorylated phosphoinositides in the cell membrane generates binding sites for the PH domain of PLCγ. Protein expression of the membrane-targeted active p110β, the kinase-inactivated membrane-targeted p110β and the dominant-interfering p85 was verified by immunoblot analysis in each case. Taken together, the experiments described above demonstrate that PtdIns(3,4,5)P3 functions as a specific ligand for the PH domain of PLCγ1 at the cell membrane.
Effect of the PH domain on PLCγ enzyme activity

To determine the effect of membrane localization on PLCγ enzymatic activity, Ins(1,4,5)P₃ production was examined in PDGF-stimulated COS-1 cells and IMR-33 cells in the presence of Wortmannin, and also in COS-1 cells that overexpress the PLCγ PH domain or a dominant-interfering mutant of p85. The experiment presented in Figure 5A shows that PDGF-induced Ins(1,4,5)P₃ production is reduced by ~65% in cells that overexpress the PH domain or are treated with 100 nM Wortmannin. Approximately 90% inhibition of PDGF-induced Ins(1,4,5)P₃ production was recorded in cells overexpressing the dominant-interfering mutant of PI 3-kinase p85. By contrast, overexpression of the PH domain mutant that does not localize to the plasma membrane or bind to PtdIns(3,4,5)P₃ (3/4 mutant) did not affect PDGF-induced stimulation of Ins(1,4,5)P₃ production (data not shown), demonstrating that the inhibition induced by the wild-type PH domain is specific. Interestingly, Wortmannin treatment did not influence the ability of PDGF to induce tyrosine phosphorylation of PLCγ (Figure 5B), indicating that PI 3-kinase does not regulate the activity of PLCγ at the level of tyrosine phosphorylation.

The experiments presented here demonstrate that growth factor-induced generation of PtdIns(3,4,5)P₃ leads to translocation of PLCγ to the plasma membrane, a step that increases the local concentration of the enzyme in the vicinity of its substrate PtdIns(4,5)P₂, probably leading to more efficient substrate hydrolysis. Activation of PLCγ in response to extracellular signals involves several regulatory steps. Following growth factor stimulation, receptor tyrosine kinases undergo autophosphorylation on tyrosine residues, which function as docking sites for the SH2 domains of PLCγ, thus providing one mechanism for targeting the enzyme to the cell surface. Membrane association is stabilized further by binding of the PH domain of PLCγ to PtdIns(3,4,5)P₃ molecules in the membrane that are generated by PI 3-kinase in response to growth factor stimulation (Figure 6). Recent experiments by Bae et al. (1988) indicate that the SH2 domains of PLCγ may also contribute to its binding to PtdIns(3,4,5)P₃, thus providing an additional anchor in the plasma membrane. Thus, several targeting signals appear to act together to generate a stable anchor for PLCγ in the plasma membrane where its substrate is found. An additional step in the activation of PLCγ involves tyrosine phosphorylation of PLCγ mediated by either receptor or non-receptor protein tyrosine kinases, which leads to activation of PLCγ by a poorly understood allosteric mechanism.

A similar molecular scenario was described recently for growth factor-induced activation of the Ser/Thr kinase Akt/PKB. It has been shown that this kinase is targeted to the plasma membrane in response to growth factor stimulation by means of the Akt/PKB PH domain binding to PtdIns(3,4)P₂. This brings Akt/PKB in proximity to a membrane-bound protein kinase which phosphorylates and...
activates Akt (Alessi et al., 1997; Stokoe et al., 1997). In this case, Akt/PKB is activated by the concerted action of PI 3-kinase and a Ser/Thr kinase. Here we demonstrate that PLCγ1 is activated by PI 3-kinase acting in concert with a receptor or non-receptor protein tyrosine kinase. In the case of PLCγ1, the activity of an enzyme that plays a crucial role in phosphoinositide metabolism is regulated by another enzyme that plays a key role in the same metabolic process (PI 3-kinase), thus revealing a previously unappreciated connection within the intricate circuitry that takes place in the control of this family of second messengers.

Materials and methods

Construction of expression plasmids

The cDNA fragments corresponding to the PH domains from rat PLC-γ1 (residues 14–150) and rat PLC-δ1 (residues 11–140) were amplified using PCR. PCR products were digested with BamHI alone (for PLCγ1-PH) or BamHI plus EcoRI (for PLCδ1-PH), depending on the sites present in the oligonucleotides used for PCR, and were ligated into the appropriately digested pGEX-2T bacterial expression vector to direct expression of GST–PH domain fusion proteins. Mutated PH domains were generated by four-primer PCR mutagenesis, as previously described (Artalejo et al., 1997). The DNA sequence of each PH domain insert was verified by dideoxynucleotide sequencing.

GFP expression vector

cDNA encoding the different PH domains was subcloned (in-frame with GFP) into the GFP fusion protein expression vector pEGFP-C1 (Clontech), using the BglII and BamHI sites for PLCγ1-PH and the BglII and EcoRI sites for PLCδ1-PH. The sequence of each insert was verified by dideoxynucleotide sequencing.

Expression and purification of PH domains

The pGEX-2T plasmid containing the wild-type or mutated PH domains was transformed into Escherichia coli DH5α. Cells were grown to an OD600 of 0.3–0.4, and induced with 1 mM isopropyl-1-thio-

β-galactopyranoside (IPTG) (Boehringer Mannheim) for 3 h at 37°C (for PLCγ1-PH), or for 4–7 h at 25°C (for PLCδ1-PH and mutants). After pelleting, cells were lysed by sonication in 50 mM Tris, pH 8.0, 100 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), containing protease inhibitors [1 mM phenylmethylsulfonylfluoride (PMSF), 10 μg/ml leupeptin and 10 μg/ml aprotinin]. Triton X-100 was added to 1% in the microinjection buffer (PBS) using a Centricon-10 (Amicon). The clarified lysate and particulates were removed by centrifugation for 20 min at 16 000 r.p.m. in an SS34 (Sorvall) rotor. The Triton lysate was incubated with glutathione–agarose beads (Sigma) for 1 h at 4°C, washed 16 000 r.p.m. in an SS34 (Sorvall) rotor. The clarified lysate was incubated with glutathione–agarose beads (Sigma) for 1 h at 4°C, washed three times with cold phosphate-buffered saline (PBS) containing 1 mM DTT plus protease inhibitors, and protein was stored at 4°C bound to the glutathione–agarose beads. For microinjection studies, protein was eluted from the beads by incubation with 15 mM reduced glutathione in PBS, and the eluted protein was buffered-exchanged into microinjection buffer (PBS) using a Centricon-10 (Amicon).

Cell culture and transfection

IMR33, L6, HeLa and COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were plated onto gridded glass coverslips. Cells were then extracted with methanol/1 M HCl/chloroform 1:1:1 (v/v/v) and analyzed by high-performance liquid chromatography (HPLC) as described (Falasca et al., 1997).

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References


