Akt Interaction with PLCγ Regulates the G₂/M Transition Triggered by FGF Receptors from MDA-MB-231 Breast Cancer Cells

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Abstract. Background/Aim: Estrogen-independent breast cancer cell growth is under the control of fibroblast growth factors receptors (FGFRs), but the role of phospholipase C gamma (PLCγ) and Akt, the downstream effectors activated by FGFRs, in cell proliferation is still unresolved. Materials and Methods: FGFRs from highly invasive MDA-MB-231 cells were expressed in Xenopus oocyte, a powerful model system to assess the G₂/M checkpoint regulation. Under FGF1 stimulation, an analysis of the progression in the M-phase of the cell cycle and of the Akt signaling cascades were performed using the phosphatidylinositol-3-kinase inhibitor, LY294002, and a mimetic peptide of the SH3 domain of PLCγ inhibitor, LY294002, and a mimetic peptide of the SH3 domain of PLCγ. Results: Activated Akt binds and phosphorylates PLCγ before Akt targets the tumor suppressor Chfr. Disruption of the Akt-PLCγ interaction directs Akt binding to Chfr and accelerates the alleviation of the G₂/M checkpoint. Conclusion: The PLCγ-Akt interaction, triggered by FGF receptors from estrogen-independent breast cancer cells MDA-MB-231, regulates progression in the M-phase of the cell cycle.

Fibroblast growth factors (FGFs) are involved in mammary gland growth (1). In breast cancer, FGFs stimulate cell proliferation and angiogenesis (2-4). The estrogen-negative human breast cancer cell line MDA-MB-231 proliferates and migrates under an autocrine secretion of FGF1 (5). The binding of FGF1 to fibroblast growth factor receptors (FGFRs) results in receptor activation and initiates several transduction cascades (6). Two important pathways, the phospholipase C gamma (PLCγ) and the phosphatidylinositol-3-kinase (PI3K/Akt pathway have been associated with breast cancer (7-10). PLCγ promotes MDA-MB-231 cell migration, invasiveness and metastasis (7, 11), whereas the PI3K/Akt pathway promotes breast cancer cell survival and resistance to several chemo- and radiotherapeutics (12, 13). An interaction between Akt and PLCγ was described to modulate cell motility under epidermal growth factor (EGF) stimulation. EGF causes conformational changes of PLCγ allowing the interaction of its SH3 domain with Akt proline-rich motifs, thus triggering serine phosphorylation and downregulation of PLCγ (14). Checkpoint with forkhead and ring finger domains (Chfr) is another Akt target, acting as a G₂/M checkpoint protein and exerting a tumour suppressor effect. Under growth factor stimulation, Akt phosphorylates and inhibits Chfr activity, releasing the G₂/M checkpoint and allowing the entry in the M-phase of the cell cycle (15-18).

Cancer cell signaling cascades are complex to decipher, partly because they are generated by the superposed action of many growth factors. To overcome this complexity, we have used a model system devoid of FGFRs, the Xenopus oocyte, a powerful experimental system to question transduction cascade effects on the G₂/M cell cycle checkpoint regulation (19). FGFRs from MDA-MB-231 can be expressed and specifically stimulated by the addition of exogenous FGF1 that induces Ras-dependent and Ras-independent cascades leading to the oocyte G₂/M transition in the cell cycle (19, 20).

To understand the connection between the PLCγ and the Akt pathways induced by FGF1-FGFR signaling and their effect on the G₂/M cell cycle progression, we thus took advantage of this powerful expression system.
Materials and Methods

Breast cancer cell culture and oocyte handling. MDA-MB-231 cells were cultured at confluence in a humidified atmosphere of 5% CO₂ in Eagle’s medium containing 10% fetal calf serum, 20 mM HEPES, 2 g/l sodium bicarbonate, 2 mM l-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 10 μg/l gentamycin sulfate and 5 μg/ml insulin.

After anaesthesia with MS 222 (1 g/l; Sandoz, Vienna, Austria), Xenopus laevis ovarian fragments were surgically removed and placed in ND96 medium (in mM: NaCl 96, KCl 2, MgCl₂ 1, CaCl₂ 1.8, HEPES 5, adjusted to pH 7.4 with NaOH), supplemented with streptomycin/penicillin (50 μg/ml; Eurobio, Les Ulis, France), sodium pyruvate (225 μg/ml; Sigma, Saint Quentin Fallavier, France) and soybean trypsin inhibitor (30 μg/ml; Sigma). Stage VI oocytes were harvested by using 1 h treatment with collagenase A (1 mg/ml, Boehringer, France). Complete defolliculation of the oocytes was achieved by manual dissection. The oocytes were kept at 19˚C in the ND96 medium.

Fusion protein and RNA preparations. The PLCγ-SH2-SH2 domains (a gift from Dr S.A. Courtneidge) and the PLCγ-SH3 (a gift from Dr. E. Kay) expressed as GST fusions were produced as described elsewhere (21). The rat Chfr cDNA sequence was cloned into the pGex4T2 expression vector (Pharmacia Biotech, Orsay, France). Chfr T39D and T39A mutations were performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) as described in (22).

PolyA mRNAs from MDA-MB-231 cells were extracted by the guanidium thiocyanate/caesium chloride gradient, using RNA plus reagent from Bioprobe (Montreuil, France) followed by polydT columns (Pharmacia) (20, 23).

Microinjection and drug treatment of oocytes. Microinjection of 60 ng of MDA-MB-231 FGFR mRNAs was performed in oocytes, 48 h before the addition of FGF1 to the extracellular medium (5 nM). One hundred ng of purified GST fusions of SH3 domain of PLCγ or Chfr mutated form T39D and T39A were injected 1 h before FGF1 stimulation; 20 μM of LY294002 (Sigma) was added 1 hour before FGF1 stimulation and remained for 15 h. For each experiment, 20-30 oocytes were removed from 3 animals.

G2/M checkpoint assay. Stage VI oocytes expressing FGFRs from MDA-MB-231 for 48 h, treated or not with inhibitors and stimulated for 15 h by FGF1 (5 nM), were analyzed for their state of progression in the cell cycle. The detection at the animal pole of a white spot attests to G2/M transition (19).

Electrophysiological measurements. Electrophysiological measurements were performed using the standard two microelectrode voltage-clamp technique (WPI instruments and Axon Instruments, Berlin, Germany) as described previously (24). Oocytes were placed in a recording chamber, impaled with 3 M KCl-filled microelectrodes (resistance <5 MΩ) and voltage clamped at –80 mV. After stabilisation of the membrane currents, the calcium-dependent chloride current was recorded. For each experiment, 3 oocytes from 3 different Xenopus were recorded.

PLCγ and Akt immunoprecipitations. Twenty oocytes expressing FGFRs from MDA-MB-231 for 48 h, treated or not with inhibitors and stimulated or not for 5 min by FGF1, were lysed in 200 μl of buffer A (25 mM 4-Morpholinepropanesulfonic acid (MOPS) pH 7.2, 60 mM β-glycerophosphate, 15 mM paraaminophenyl phosphate, 15 mM EDTA, 15 mM MgCl₂, 2 mM diithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM NaF, 1 mM phenylphosphate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, 10 μM benzamidine) and centrifuged for 10 min at 10,000×g and 4˚C. Pellets were vigorously resuspended in 200 μl of buffer A added with 1% Triton X-100. After a 15 min centrifugation at 10,000×g and 4˚C, supernatants were precleared with protein A-agarose (Sigma) and centrifuged for 1 h at 4˚C. Immunoprecipitations were performed using anti-PLCγ (Upstate Biotechnology, Dundee, UK), or anti-Akt1 antibodies (Santa Cruz Biotechnology, Santa Cruz, USA) for 3 h and Protein A-agarose (Sigma) for 1 h at 4˚C. Immunocomplexes were collected by centrifugation, rinsed three times, resuspended in Laemmli sample buffer and subjected to SDS-PAGE analysis. Electrophoresis and Western blot analysis. For Western blot analysis, oocyte lysates were electrophoresed and proteins were
transferred as described elsewhere (21). The membranes were treated with the following antibodies: anti-PLCγ (Upstate Biotechnology), anti-phospho-serine-PLCγ (Invitrogen, CA, USA) or anti-phospho-PLCγ (Tyr 783; Upstate Biotechnology), anti-FGFR1 (clone 19B2; Upstate Biotechnology), anti-Akt1 (Santa Cruz), anti-phospho-Akt (Ser 473; Upstate Biotechnology), anti-phospho-Chfr (a gift from E. Shtivelman), anti-Xenopus-Chfr (a gift from A. Castro). Detection of the immunocomplexes was performed by an advanced ECL detection system (Amersham, Buckinghamshire, UK).

Results

The G2/M transition triggered by MDA-MB-231 FGFRs depends on Akt and PLCγ signaling pathways. FGF1 stimulation of oocytes expressing FGFRs from MDA-MB-231 for 48 h induced the G2/M transition. An inhibitor of the PI3K/Akt pathway, LY294002 and an inhibitor of PLCγ, a PLCγ SH2-SH2 domain peptide that impedes the direct binding to FGFR p(Y)766 site (16), added 1 h before stimulation with FGF1, both suppressed the G2/M transition (Figure 1A). Disruption of the Akt-PLCγ interaction using an SH3 domain of PLCγ, which binds to the proline-rich region located in the N-terminal region of Akt, significantly accelerated oocyte exit from the G2 phase (548 ± 38.5 min, compared to 684 ± 20.7 min, n=3 in FGFR control, p<0.01) (Figure 1A and 1B). A significantly earlier G2/M transition also occurred when a phosphorylated form of Chfr (T39D mutant) (544±10.3 min) was microinjected, as compared to the mutated unphosphorylated form of Chfr (T39A mutant) (666±15.8 min) (Figure 1A and 1B).

The Akt-PLCγ interaction controls the G2/M checkpoint alleviation. A PLCγ/calcium-dependent chloride current, measured by electrophysiological techniques, started 2 to 4 min after FGF1 addition, and lasted a duration of 30 min with a current amplitude of 146.3±49.07 nA (n=3) (Figure 2B), compared to the unstimulated controls (Figure 2A). Kinetic analysis of PLCγ phosphorylation showed that PLCγ was first phosphorylated on its tyrosine residue Y783 5 min after FGF1 stimulation (Figure 2C, lane 2) and then on serine after 30 min (Figure 2C, lane 4). Akt phosphorylation
was detected after 30 min (Figure 2C, lane 4) and further increased after 4 h (Figure 2C, lane 6). Phosphorylation of Chfr on its T39 residue, which is an Akt target, was apparent after 30 min of FGF1 stimulation.

Co-immunoprecipitation experiments provided evidence that phosphorylated Akt (serine 473) interacted with PLCγ only when it was phosphorylated on both tyrosine and serine residues (Figure 3A and 3B, compare lane 2 and lane 3). A PLCγ peptide mimicking the SH3 domain prevented the serine phosphorylation of PLCγ and thus its binding to Akt, and simultaneously favoured Akt binding to another downstream target, phosphorylated Chfr (Figure 3A and 3B, lane 5). Treatment with LY294002 inhibited Akt activation, PLCγ phosphorylation on serine residues, and Akt binding to PLCγ and Chfr (Figure 3A and 3B, lane 7).

Discussion

In the present paper we investigated the control by FGF of the G2/M transition following MDA-MB-231 FGFR signaling and Akt activation, using the Xenopus oocyte as a biological cell cycle model system (19).

As shown by the inhibitory effect of LY294002 (present study), wortmannin and the N-terminal SH2 domain of p85 PI3K used in a former study (23), which all impair the PI3K/Akt pathway, and by the effect of the addition of the tandem SH2-SH2 domains of PLCγ that blocked PLCγ binding to the FGFRs (21), both the PI3K/Akt and the PLCγ pathways are required to allow the G2/M transition triggered by MDA-MB-231 FGFRs.

We thereafter analysed the time course of Akt activation and binding to PLCγ. Following FGF1 stimulation, PLCγ was rapidly tyrosine phosphorylated and its activity was recorded by measuring the calcium-dependent chloride current which was generated through the hydrolysis of phosphoinositide biphosphate 5 min after FGF1 addition. Akt was slightly phosphorylated on its serine residue S473, a prerequisite for its activation (25), 30 min after FGF1 addition and became strongly phosphorylated after 4 h. The kinetics of Akt activation tightly correlated with the serine-phosphorylation status of PLCγ, which remained low after 30 min, but was more pronounced after 4 h. Phosphorylation of Chfr, known as a direct Akt target (17), was detected only after 4 h of FGFR stimulation, when Akt was fully phosphorylated. Co-immunoprecipitations that allowed identification of binding partners revealed that the highly phosphorylated form of Akt was bound to PLCγ, suggesting that Akt was the serine/threonine kinase that phosphorylated PLCγ. This was further confirmed by competition experiments using the SH3 domain of PLCγ, since this domain disrupted both Akt-PLCγ interaction and PLCγ serine-phosphorylation. The PI3K inhibitor, LY294002, which impeded Akt activation, also abolished PLCγ serine phosphorylation and its binding to Akt. Importantly, we showed that the disruption of the Akt-PLCγ interaction using the SH3 domain of PLCγ, expedited the cell cycle M-phase entry. This disruption increased Akt phosphorylation and induced Akt binding to the non-canonical ubiquitine ligase Chfr and phosphorylation of this substrate. An accelerated G2/M checkpoint alleviation, displaying kinetics similar to that observed in the presence of the SH3 domain of PLCγ, was reproduced by overexpressing the mutated phosphorylated form of Chfr, ChfrT39D.
In conclusion, our data suggest a model whereby FGF-induced cell cycle progression into the M-phase in the estrogen-independent breast cancer line MDA-MB-231 is controlled by an ordered interaction of activated Akt with its substrates PLCγ and Chfr. Akt phosphorylates PLCγ before Chfr, which allows the cell cycle checkpoint release. The disruption of the Akt interaction with PLCγ forces Akt binding to Chfr, the phosphorylation of Chfr by Akt and promotes an earlier G2/M transition. As a consequence, antiproliferative therapeutic strategies targeting Akt should maintain this Akt-PLCγ interaction to avoid an accelerated M-phase entry.

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References


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