Journal of Cellular Biochemistry 72:339–348 (1999)

Insulin-Like Growth Factor-I-Dependent Stimulation of Nuclear Phospholipase C-β1 Activity in Swiss 3T3 Cells Requires an Intact Cytoskeleton and is Paralleled by Increased Phosphorylation of the Phospholipase

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Abstract Swiss 3T3 mouse fibroblasts were exposed to 10 μM colchicine to disrupt microtubules, then stimulated with insulin-like growth factor-I. Immunoprecipitation experiments showed that insulin-like growth factor-I receptor and insulin receptor substrate-1 were tyrosine phosphorylated to the same extent in both cells treated with colchicine and in those not exposed to the drug. Moreover, the activity of phosphatidylinositol 3-kinase was not affected by incubation with colchicine. While in nuclei prepared from cells not exposed to colchicine it was possible to detect an insulin-like growth factor-I-dependent increase in the mass of diacylglycerol, as well as stimulation of phospholipase C activity, no similar changes were observed in nuclei obtained from cells treated with colchicine. Activation of the nuclear phospholipase activity was paralleled by an increase of its phosphorylation. Immunofluorescent studies revealed that mitogen-activated protein kinase did not translocate towards the nucleus when the cytoskeleton was depolymerized. These results show that in Swiss 3T3 cells some as yet unknown events necessary for the insulin-like growth factor-I-dependent activation of nuclear polyphosphoinositide metabolism require the presence of an intact cytoskeleton and are situated down-stream the activation of insulin receptor substrate-1 and phosphatidylinositol 3-kinase. Activation of nuclear phospholipase C-β1 might be linked to its phosphorylation and translocation of mitogen-activated protein kinase to the nucleus. J. Cell. Biochem. 72:339–348, 1999.

Key words: polyphosphoinositide cycle; phospholipase C-β1; nucleus; cytoskeleton; depolymerization; insulin-like growth factor-I; phosphorylation; mitogen-activated protein kinase

It is now well-established that nuclei have a polyphosphoinositide cycle distinct from, and under a separate control, the "classic" cycle located at the plasma membrane [Cocco et al., 1994]. The existence of such a nuclear cycle has been demonstrated in several cell lines and tissues [Divecha et al., 1991, 1993; Banfic et al., 1993; York and Majerus, 1994; York et al., 1994; Marmiroli et al., 1994; Neri et al., 1997]. Compelling evidence suggests this cycle to be involved in the control of both cell proliferation and differentiation [Martelli et al., 1992, 1994; Zini et al., 1993, 1995; Divecha et al., 1995; Rubbini et al., 1997; Bertagnolo et al., 1997; Sun et al., 1997]. A key enzyme of this cycle is phospholipase (PLC)-β1, that, in Swiss 3T3 mouse fibroblasts, appears to be mainly localized within the nucleus [Martelli et al., 1992; Zini et al., 1993]. Recently, it has been demonstrated that in Swiss 3T3 cells this nuclear enzyme plays an essential role in controlling insulin-like growth factor-I (IGF-I)-promoted mitogenesis [Manzoli et al., 1997]. No information at all is at present available concerning

Abbreviations used: BSA, bovine serum albumin; DAG, diacylglycerol; FITC, fluorescein isothiocyanate; h.p.l.c., high performance liquid chromatography; IGF-I, insulin-like growth factor I; IP_3, inositol 1,4,5 trisphosphate; IRS-1, insulin receptor substrate-1; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase kinase; PKB, protein kinase B; PKC, protein kinase C; PI, phosphatidylinositol; PI 3-K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5 bisphosphate; PLC, phospholipase C; PMSF, phenylmethylsulfonyl fluoride.

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Received 12 June 1998; Accepted 29 July 1998

how a growth factor, interacting with a receptor located at the plasma membrane level, could stimulate a polyphosphoinositide cycle present within the nucleus. Evidence suggests that the nucleus is connected with the cell periphery through interactions mediated by elements of the cytoskeleton [Pienta and Coffey, 1992; Maniotis et al., 1997]. Recently, Schmalz et al. [1996] have demonstrated that in NIH 3T3 cells phorbol ester-promoted nuclear translocation of PKC-α requires an intact cytoskeleton. Thus, we sought to determine whether or not an intact cytoskeleton might be required to allow activation of the nuclear polyphosphoinositide cycle. In this report we show that in Swiss 3T3 cells depolymerization of microtubule network by colchicine inhibits IGF-I-dependent activation of nuclear PLC-β1 [Martelli et al., 1992] as well as the increase in the mass of nuclear diacylglycerol (DAG) that is seen after treatment with this growth factor [Divecha et al., 1991]. On the contrary, other cellular events that are known to be induced by IGF-I (i.e., tyrosine phosphorylation of the β subunit of the IGF-I receptor and of insulin receptor substrate [IRS-1] as well as activation of phosphatidylinositol 3-kinase [PI 3-K] activity) are not inhibited by treatment with colchicine. Treatment of cells with colchicine also prevented the transient hyperphosphorylation of nuclear PLC-β1 which parallels IGF-I-dependent activation of the enzyme. Moreover, in cells with a depolymerized cytoskeleton, IGF-I exposure did not result in translocation to the nucleus of the mitogen-activated protein kinase (MAP kinase). These results strengthen the contention that the cytoskeleton plays a very important role in conveying signals from the cell periphery to the nucleus and point to the likelihood that activation of nuclear polyphosphoinositide cells in Swiss 3T3 fibroblasts exposed to IGF-I requires an increased phosphorylation of PLC-β1 and nuclear translocation of MAP kinase.

MATERIALS AND METHODS

Materials

D-MEM, fetal calf serum, colchicine, 1,2-dioleyl-3-palmitoyl-glycerol, phosphatidylinositol (PI), CHAPS, normal rabbit IgG, normal goat serum, peroxidase-conjugated anti-mouse or anti-rabbit IgG, fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG, Cy3-conjugated anti-mouse IgG, and bovine serum albumin (BSA) were from Sigma Chemical Company (St. Louis, MO). IGF-I and the enhanced chemiluminescence detection kit were from Boehringer Mannheim (Germany). Protein A-Agarose was purchased from Transduction Laboratories. [^{3}H] phosphatidylinositol 4,5-bisphosphate ([^{3}H] PIP_2), [γ-^{32}P] ATP, and [^{32}P] ortophosphate were from Amersham International (Buckinghamshire, UK). The Protein Assay kit (detergent compatible) was from Bio-Rad (Richmond, CA). The following antibodies were used in this study: monoclonal antibodies to β-tubulin and to phosphotyrosine (clone PT-66; Sigma); rabbit polyclonal antibodies to IGF-I receptor, IRS-1, and to the 85-kDa subunit of PI 3-K (Upstate Biotechnology Incorporated, Lake Placid, NY); monoclonal antibody to MAP kinase (Transduction Laboratories, Lexington, KY); rabbit polyclonal antibody to PLC-β1 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Culture

Swiss 3T3 mouse fibroblasts were cultured in D-MEM containing 10% fetal calf serum. Prior to stimulation, cells were subcultured at a density of 10^4/cm, and incubated until they became confluent (6 days). They were then cultured for an additional 24 h in serum-free medium containing 0.5% BSA. Quiescent cultures were washed twice with serum-free medium containing 0.2% BSA, then incubated in the same medium for the indicated times in the presence of 50 ng/ml of IGF-I. For treatment with colchicine, the drug (dissolved in water) was added at 10 μM to serum-free medium for 1 h prior to stimulation with IGF-I.

Immunocytochemistry

Immunofluorescent staining for β-tubulin, and MAP kinase was performed essentially as reported by Zini et al. [1993], using the respective primary antibodies diluted 1:100, followed by the appropriate secondary antibody conjugated to FITC or Cy3. Samples were photographed using a Zeiss Axiophot epifluorescence microscope (Zeiss, Inc., Thornwood, NY).

Immunoprecipitation

Cell were washed with PBS, then scraped and lysed for 30 min at 4°C in 50 mM Hepes, pH 7.9, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.0 mm Na_3VO_4, 4 mM sodium pyrophosphate, 10 mM NaF, 1 mM phenyl-

methylsulfonyl fluoride (PMSF), 1 μg/ml leupeptin and aprotinin. The suspension was passed several times through a 26-gauge needle, then centrifuged at 12,000*g* for 15 min at 4°C. Cell lysates (1 ml, containing 500 μg of protein) were pre-cleared by adding 5 μg of normal rabbit IgG and 10 μg of 50% Protein A-Agarose, followed by incubation for 1 h at 4°C and centrifugation at 12,000*g* for 10 min at 4°C. Cell lysates were incubated for 2 h at 4°C under constant agitation with 5 μg of polyclonal antibodies to either IGF-I receptor or IRS-1. 10 μg of 50% Protein A-Agarose was added and incubation proceeded for 1 h at 4°C under constant agitation. The immunoprecipitates were washed three times with lysis buffer, then resuspended in electrophoresis sample buffer.

Western Blot Analysis

Proteins separated on 7.5% polyacrylamide gels [Laemmli, 1970] were transferred to nitrocellulose sheets using a semi-dry blotting apparatus (Hoefer/Pharmacia Biotech, Uppsala, Sweden). Sheets were saturated in PBS containing 5% normal goat serum and 4% BSA for 60 min at 37°C (blocking buffer), then incubated overnight at 4°C in blocking buffer containing primary antibodies. After four washes in PBS containing 0.1% Tween-20, they were incubated for 30 min at room temperature with peroxidase-conjugated anti-mouse or anti-rabbit IgG, diluted 1:3,000 in PBS-Tween-20, and washed as above. Bands were visualized by the enhanced chemiluminescence method.

Isolation of Nuclei

This was accomplished as previously reported [Martelli et al., 1992]. Briefly, cells (5×10^6) were suspended in 500 μl of 10 mM Tris-Cl, pH 7.8, 1% NP-40, 10 mM β-mercaptoethanol, 0.5 mM PMSF, 1 μg/ml of leupeptin and aprotinin for 2 min at 0°C. Then 500 μl of double-distilled H_20 was added and the cells allowed to swell for 2 min. Cells were sheared by 10 passages through a 22-gauge needle. Nuclei were recovered by centrifugation at 400*g* for 6 min and washed once in 10 mM Tris-Cl, pH 7.4, 2 mM $MgCl_2$, plus protease inhibitors as above.

DAG Measurement

The assay was performed according to Divecha et al. [1991], using DAG kinase enzyme purified from rat brain. DAG was extracted from nuclei as reported earlier [Divecha et al., 1991], dissolved in 20 μl of CHAPS (9.2 mg/ml) and sonicated at room temperature for 15 sec. After the addition of 80 μl of reaction buffer (50 mM Tris-acetate, pH 7.4, 80 mM KCl, 10 mM Mg acetate, 2 mM EGTA), the assay was started by the addition of 20 μl of DAG kinase enzyme followed by 80 μl of reaction buffer containing 5 μM ATP, and 1 μCi of [γ-^{32}P] ATP. Incubation was for 1 h at room temperature, then phosphatidic acid was extracted, chromatographed, autoradiographed, and its radioactivity counted as reported elsewhere [Divecha et al., 1991]. Standard curves were obtained as reported by Divecha et al. [1991], using 1,2-dioleyl-3-palmitoyl-glycerol as substrate.

PLC Activity Assay

The procedure outlined by Martelli et al. [1992] was followed. Assays (100 μl) contained 100 mM MES buffer, pH 6.7, 150 mM NaCl, 0.06% sodium deoxycholate, 3 nmol [^{3}H] PIP_2 (specific activity 30,000 dpm $nmol^{-1}$), 10 μg of nuclear protein. Incubation was for 30 min at 37°C. Hydrolysis was stopped by adding chloroform-methanol-HCl, and inositol 1,4,5-trisphosphate (IP_3) recovered from the aqueous phase was analyzed by h.p.l.c., using a Partisil 10 SAX column eluted with a linear gradient from distilled water to 2 M ammonium formate (pH 3.7, adjusted with phosphoric acid). Fractions (1 ml) were collected and counted by liquid scintillation using a Betamatic IV scintillation counter (Kontron, Milan, Italy).

PI 3-K Activity Assay

Cells were lysed in 50 mM Hepes, pH 7.4, 10 mM EDTA, 2.0 mm Na_3VO_4, 10 mM sodium pyrophosphate, 10 mM NaF, 1% NP-40 for 15 min at 4°C, and immunoprecipitation of PI 3-K was performed as described above using polyclonal antiserum to the 85-kDa regulatory subunit [Zauli et al., 1997]. The immunoprecipitates were washed twice with each of the following buffers: 1) PBS, pH 7.4, containing 1% NP-40; 2) 100 mM Tris-Cl (pH 7.4), 0.5 M LiCl; 3) 10 mM Tris-Cl, pH 7.4, 100 mM NaCl, 1 mM EDTA [Harada et al., 1996]. The PI 3-K activity assay was then performed by adding sonicated PI (0.5 mg/ml in 10 mm Hepes, pH 7.5, 1 mM EDTA), 10 mM $MgCl_2$, and 50 μM [γ-^{32}P] ATP (10 Ci/mmol). Incubation was for 15 min at room temperature. Phosphoinositides were extracted, analyzed, autoradiographed,

and counted as reported earlier [Zauli et al., 1997].

In Vivo Labeling With [^{32}P] Orthophosphate

Cells were labeled for 6 h in phosphate-free D-MEM in the presence of 200 μCi/ml [^{32}P] orthophosphate. Nuclei (from 2×10^7 cells) were then isolated from control and IGF-I-treated cells in buffers containing 2.0 mM Na_3VO_4 and 5 mM NaF, lysed as described elsewhere [Martelli et al., 1992], and immunoprecipitation was carried out with anti-PLC-β1 antibodies, as detailed above. Samples were then electrophoresed, blotted, and autoradiographed as described above.

Protein Assay

This was performed according to the instruction of the manufacturer using the Bio-Rad Protein Assay (detergent compatible).

RESULTS

As shown in Figure 1 immunofluorescent staining for β-tubulin revealed that after 1 h exposure to 10 μM colchicine, the microtubules of Swiss 3T3 cells were completely depolymerized.

We next performed experiments to determine whether cellular events that are known to be elicited by exposure to IGF-I were affected by incubation with colchicine. As shown in Figure 2A, after 1 min of IGF-I treatment, the levels of tyrosine phosphorylation of the β subunit of the IGF-I receptor were unaffected by pre-incubation of cells with colchicine and the same was true for tyrosine phosphorylation of IRS-1, as seen after 4 min of IGF-I treatment (see Fig. 2B). Moreover, as shown in Table 1, the stimulation of PI 3-K activity (measured after 5 min of IGF-I treatment) was essentially the same both in control (untreated) and in colchicine-treated cells.

In Figure 3 we show that IGF-I treatment of quiescent 3T3 cells induced an increase in the mass of nuclear DAG, as described earlier [Divecha et al., 1991]. Indeed, there was a nearly three-fold (2.85) increase of DAG after 5 min of stimulation and the levels stayed high up to 30 min following stimulation, then after 60 min they returned to control levels (data not shown), in agreement with Divecha et al. [1991]. However, in cells exposed to colchicine prior to IGF-I stimulation such an increase in the mass of nuclear DAG was not detected.

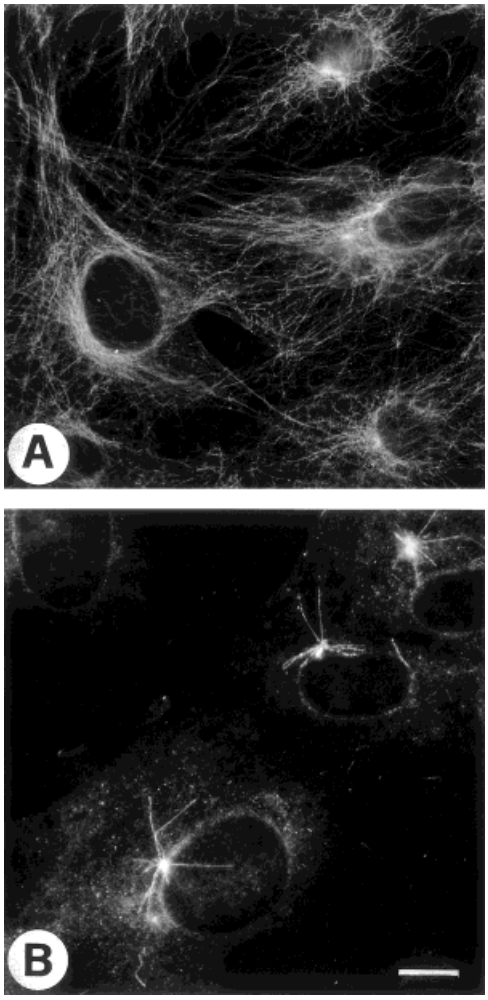

Fig. 1. Immunofluorescent staining with anti-β tubulin antibody of control (**A**) and colchicine-treated (10 μm for 1 h) Swiss 3T3 cells. A FITC-conjugated secondary antibody was employed. Scale bar = 5 μm.

In Figure 4 we show that after stimulation with IGF-I, nuclear PLC activity (expressed as nmoles of IP_3 liberated) also increased, in agreement with our own previous data [Martelli et al., 1992]. After 5 min of stimulation with IGF-I, nuclear PLC activity raised about 3.5-fold above control level. Similar levels of activity were observed up to 15 min of stimulation, then a decrease was seen and after 30 min the activity returned to basal level. On the contrary, in nuclei prepared from cells treated with colchicine prior to IGF-I stimulation, we did not measure any increase in PLC activity.

To rule out the possibility that colchicine might exert any adverse effect on nuclear PLC,

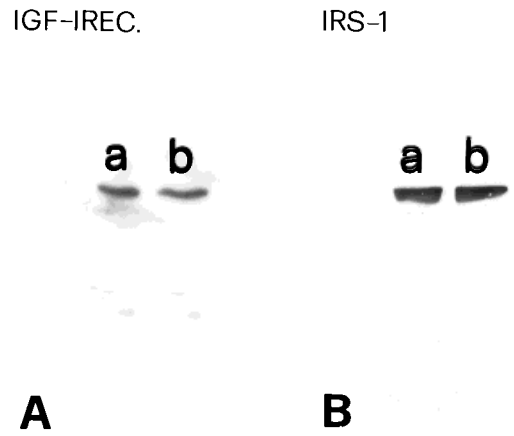

Fig. 2. Immunoprecipitation and tyrosine phosphorylation of IGF-I receptor (**A**) and IRS-1 (**B**), after 1 min and 4 min of IGF-I treatment, respectively. **Lane a**: Cells not exposed to colchicine; **lane b**: cells exposed to colchicine. Immunoprecipitated β-subunit of the IGF-I receptor banded at about 97–100-kDa, while immunoprecipitated IRS-1 migrated around 182–185-kDa.

TABLE I. Immunoprecipitation of PI 3-K Activity in Swiss 3T3 Cells After 5 Min of IGF-I Treatment[a]

Condition	cpm/assay
Control cells (unstimulated)	3,150
IGF-I-stimulated cells (10 min)	7,458
IGF-I-stimulated cells (10 min) plus colchicine	7,256

[a]Results are the mean of three different preparations with a sd > 13%.

PLC activity assays were also performed in the presence of the drug (10 μM). However, nuclear PLC activity measured in the presence of colchicine was equal to 22.4 ± 3.6 (mean ± sd, n = 3) nmoles IP_3 liberated/mg protein, while in the absence of the drug it was 23.1 ± 4.0 nmoles.

We next investigated by Western blotting analysis whether nuclei isolated from cells exposed to colchicine contained the same amount of PLC-β1 as control cells. As shown in Figure 5 it was evident that unstimulated cells (not exposed to colchicine) and IGF-I- exposed cells (both treated and untreated with colchicine) retained the same amount of protein.

Since examination of PLC-β1 sequence [Bahk et al., 1994] reveals the existence at amino acids 980–983 of a putative MAP kinase consensus sequence, P-S-S-P, we investigated whether, after IGF-I stimulation, nuclear PLC-β1 was phosphorylated. Cells were labeled in vivo with [^{32}P] orthophosphate and nuclei were isolated and lysed. PLC-β1 immunoprecipitation was then carried out, followed by electrophoresis, blotting, and autoradiography. As shown in Figure 6, PLC-β1 immunoprecipitated from control nuclear lysates was phosphorylated (lane a). At 5 min after stimulation with IGF-I an increased phosphorylation of PLC-β1 was detected (lane c).

Phosphorylation levels then decreased as observed at 25 and 30 min, respectively (Fig. 6, lanes d and e). By contrast, very low levels of phosphorylation were seen in nuclei isolated from cells that had been exposed to colchicine prior to a 5 min stimulation with IGF-I (Fig. 6, lane b). If the blots were reacted with an anti-phosphotyrosine antibody, no bands were seen corresponding to the bands immunoprecipitated by the anti-PLC-β1 antiserum, thus suggesting that the phosphorylation took place in serine/threonine residues (data not presented).

Finally, we wanted to study if translocation to the nucleus of MAP-kinase was elicited by IGF-I in 3T3 fibroblasts, and whether or not it could by abolished by colchicine pre-treatment.

In Figure 7, we demonstrate that an antibody to MAP kinase stained the cytoplasm of quiescent 3T3 cells (Fig. 7A). However, after a 10 min stimulation with IGF-I a diffuse nuclear labeling became apparent, while nucleoli were unstained (Fig. 7B). On the other hand, no nuclear staining was detected if cells had been pretreated with colchicine (Fig. 7C).

DISCUSSION

Binding of IGF-I to its receptor elicits a cascade of events such as autophosphorylation of the β subunit of the receptor itself, tyrosine phosphorylation of IRS-1 and Shc which in turn binds to SH2 domains of cytoplasmic proteins such as 85-kDa regulatory subunit of PI 3-kinase, GRB-2, and syp [Harada et al., 1996]. This is followed by activation of PI 3-kinase, MAP kinase, and protein kinase B (PKB) [e.g., Giorgetti et al., 1993; Takahashi et al., 1997; Scrimgeour et al., 1997; Parrizas et al., 1997; Kulik et al., 1997]. In Swiss 3T3 fibroblasts, IGF-I treatment also results in stimulation of nuclear PLC-β1 activity and phosphoinositide metabolism [Cocco et al., 1993]. No information at all is at present available concerning the possible mechanism(s) controlling activation of nuclear inositol lipid cycle. Since the nucleus is connected to the cell periphery through elements of the cytoskeleton, we sought to determine if an intact cytoskeleton is required for

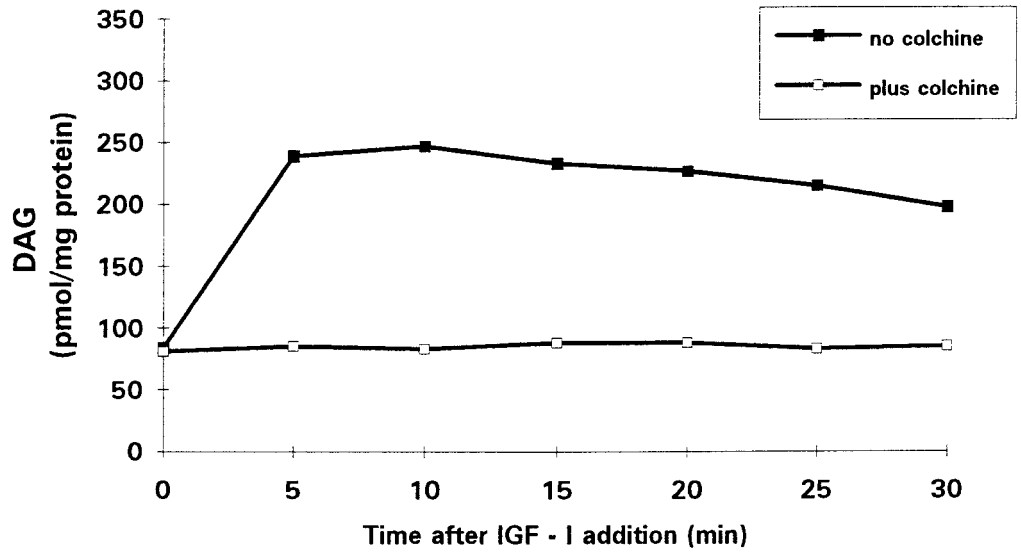

Fig. 3. Time course of changes in nuclear DAG following treatment with IGF-I in control and colchicine-incubated Swiss 3T3 cells. Results are the mean of three different preparations with a sd >16%.

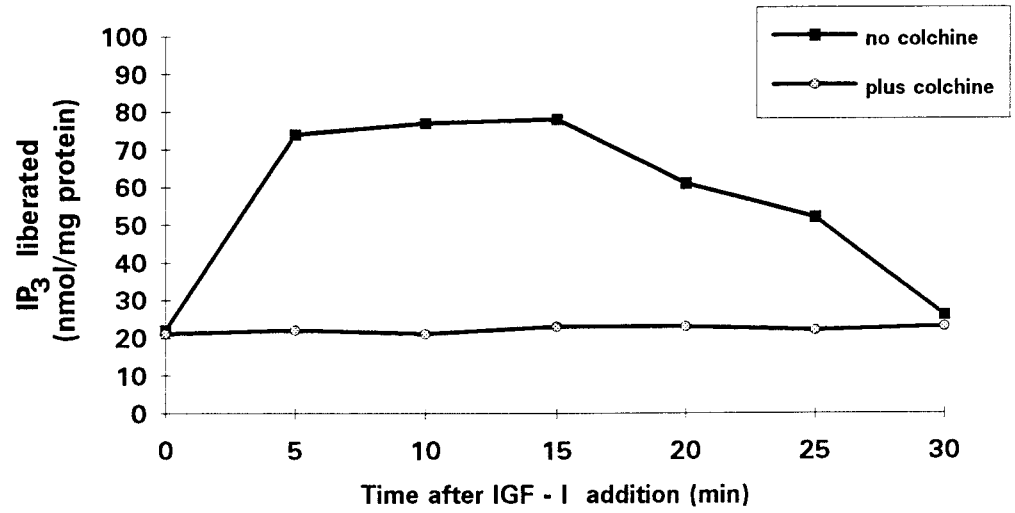

Fig. 4. Time course of nuclear PLC activity following treatment with IGF-I in control and colchicine-incubated Swiss 3T3 cells. Results are the mean of three different preparations with a sd >18%.

stimulation of nuclear PLC-β1 activity. In this paper we have demonstrated that depolymerization of the microtubule network is sufficient to abolish activation of nuclear PLC-β1 caused by IGF-I treatment. In this connection it should be reminded that disruption of one of the major elements of the cytoskeleton also markedly affects the integrity of the others [Schmalz et al., 1996]. Thus, it might be that activation of nuclear phosphoinositide cycle caused by IGF-I requires intermediate filaments that are know to be attached to the nucleus [Schmalz et al., 1996]. It should be reminded that recently Fricker et al. [1997] have identified in nuclei of many cell types (including 3T3 cells) tubular membrane-bound invaginations of the nuclear envelope which interestingly also contain elements of the cytoskeleton. Although the authors speculated the channels may be involved in the transport of molecules from the nucleus to the cytoplasm they could also serve to permit passage of molecules in the opposite direction. At the plasma membrane level, activation of PLC-β isozymes is controlled by Gq subfamily members of heterotrimeric proteins, α_q, α_{11}, α_{14}, α_{16} [Lee and Rhee, 1995; Rhee and Bae, 1997].

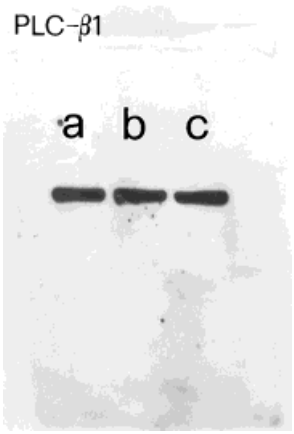

Fig. 5. Western blot analysis of nuclear PLC-β1. **Lane a**: Control cells (unstimulated and not exposed to colchicine); **lane b**: cells treated for 5 min with IGF-I but not exposed to colchicine; **lane c**: cells exposed to colchicine and treated for 5 min with IGF-I. The approximate molecular weight of the immunostained band was 150-kDa.

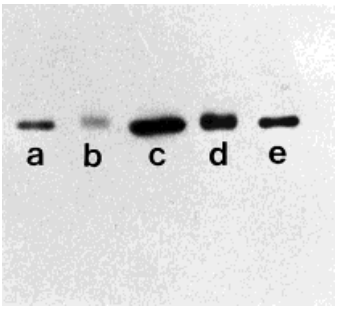

Fig. 6. In vivo phosphorylation of nuclear PLC-β1. ^{32}P-labeled nuclear PLC-β1 was immunoprecipitated, electrophoresed, blotted, and autoradiographed. **Lane a**: Control cells. **Lane b**: colchicine pre-treated cells stimulated for 5 min with IGF-I; **lanes c–e**: cells stimulated with IGF-I for 5, 25, and 30 min, respectively.

Although G-proteins have been demonstrated to reside in the nucleus [Crouch, 1991; Saffitz et al., 1994] and they have been shown to be involved in vitro in the regulation of nuclear phosphoinositide kinases [Martelli et al., 1996], we have been unable to stimulate in vitro nuclear PLC-β1 activity by GTP-γ-S or AlF4 [A.M. Martelli, unpublished experiments]. On the contrary, our results indicate that nuclear PLC-β1 is hyperphosphorylated after IGF-I treatment (5 min) of Swiss 3T3 cells, and the hyperphosphorylation parallels enzyme activation. Disruption of the microtubule network abolishes PLC-β1 hyperphosphorylation. It is established that PLC-β is phosphorylated in vitro at serine 887 by PKC [Ryu et al., 1990] and this does not result in any concomitant effect on phosphodiesterase activity. However,

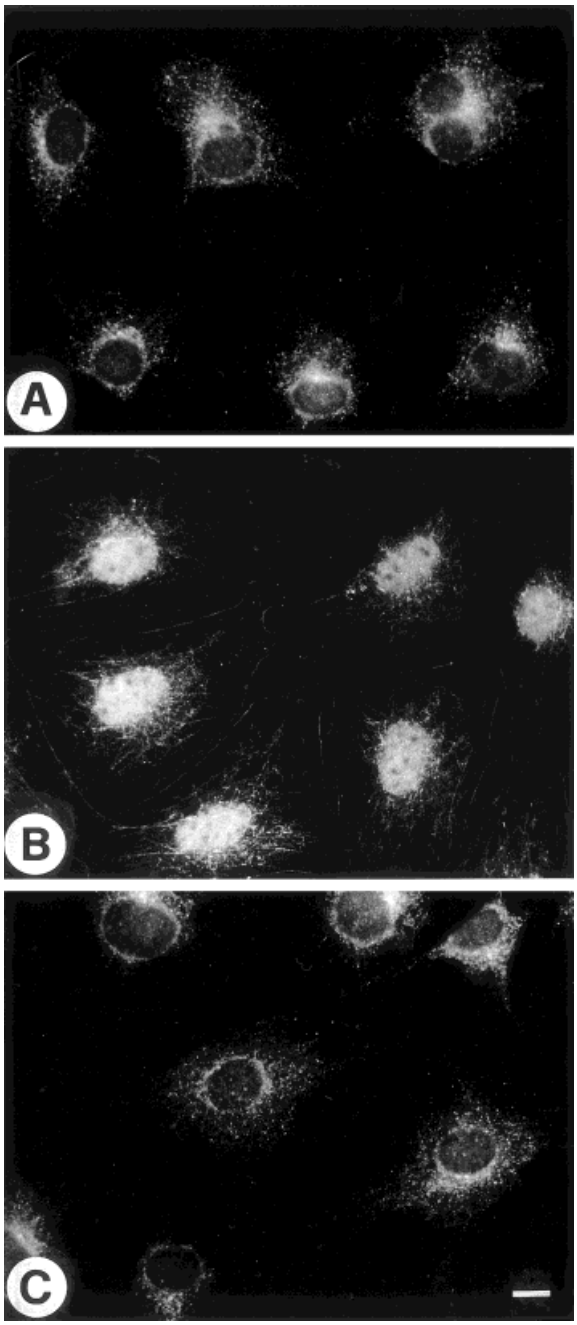

Fig. 7. Fluorescent immunostaining for MAP kinase in Swiss 3T3 cells. **A**: Quiescent cells. **B**: Cells stimulated for 10 min with IGF-I. **C**: Colchicine pre-treated cells stimulated for 10 min with IGF-I. Translocation of MAP kinase to the nucleus (B) was detected in 26% of cells (sd ± 5, n = 3). A Cy3-conjugated secondary antibody was employed. Scale bar = 5 μm.

treatment of a variety of cells with phorbol esters, which are powerful activators of PKC, results in the inhibition of receptor-coupled, phosphoinositide-specific, phospholipase activity. Therefore, it has been suggested that phos-

phorylation of PLC-β by PKC may alter its interaction with G-proteins, thereby preventing activation of PLC. This is at variance with our results, because we have presented evidence suggesting that activation of nuclear PLC-β1 requires its hyperphosphorylation. Since disruption of microtubule network prevented translocation of MAP kinase to the nucleus, this kinase is a likely candidate for the increased phosphorylation of nuclear PLC-β1, also taking into account the fact the PLC-β1 has a putative MAP kinase consensus sequence and that IGF-I treatment of Swiss 3T3 cells results in the activation of MAP kinase [Hansson and Thorén, 1995]. Also hyperphosphorylation of nuclear PLC-β1 might affect its interaction(s) with other protein(s), thus allowing its activation. In the future, it will be interesting to investigate whether or not PLC-β1 is an in vitro substrate for purified MAP kinase. According to Schmalz et al. [1996] disruption of the cytoskeleton prevented translocation to the nucleus of PKC-α, which does not possess a canonical nuclear localization sequence, while intranuclear transport of a protein with such a sequence was not affected by colchicine exposure. Thus, our results are in agreement with theirs, because also MAP kinase does not have a classic nuclear localization sequence [Lenormand et al., 1993]. It should be reminded that activation of MAP kinase requires its phosphorylation on Tyr-185 and Thr-183 by upstream dual-specific protein kinase, MAP kinase kinase (MEK). Full activation of MEK also requires phosphorylation by an upstream protein kinase on its serine and threonine residues, and several serine/threonine kinases have been shown to phosphorylate and activate MEK, including MEK kinase, c-Raf, and c-Mos [Kim and Khan, 1997]. Thus, disruption of cytoskeleton may prevent activation of MAP kinase by MEK. In this connection it should be emphasized that a small pool of microtubule-associated MAP kinase has been identified in hippocampal neurons and PC12 cells [Morishima-Kawashima and Kosik, 1996]. Moreover, a member of the MEK family, MEK5, possesses a 23 amino acid stretch similar to a sequence found in proteins believed to associate with actin [English et al., 1995].

Nevertheless, it might be that the hyperphosphorylation of nuclear PLC-β1 depends on other protein kinases translocating from the cytoplasm to the nucleus, for example PKB, or on as yet unknown signals from the cell periphery which activate a protein kinase already resident in the nucleus. PKB, also known as c-AkT, is activated rapidly when mammalian cells are stimulated with growth factors, and much of the current interest in this enzyme stems from the observation that it lies downstream of PI 3-kinase on intracellular signaling pathways [Alessi et al., 1997]. PKB translocates to the nucleus upon its activation by growth factors [Meier et al., 1997]. Interestingly, PKB does not have a canonical nuclear localization sequence. Our preliminary results reveal that microtubule network disruption by colchicine also inhibits its IGF-I-promoted nuclear translocation of PKB [A.M. Martelli, unpublished experiments].

Since IGF-I exerts a plethora of effects including mitogenesis, tumorigenesis, and inhibition of apoptosis it is conceivable that multiple signal pathways are activated by this growth factor [e.g., Harada et al., 1996; Scrimgeour et al., 1997; Kooijman et al., 1995; Valentinis et al., 1997] which are becoming to be at least partially unraveled. We feel that studies of the events taking place at the cell periphery should provide an important insight to clarify the mechanisms causing an activation of nuclear phosphoinositide metabolism following IGF-I treatment of Swiss 3T3 cells. For example, recent results obtained using IGF-I receptors carrying different mutations at tyrosine residues have demonstrated that the domains of the IGF-I receptor required for its antiapoptotic function are distinct from those required for its proliferation or transformation functions [O'Connor et al., 1997]. Similar investigations should add further to our knowledge of the mechanisms responsible for stimulation of nuclear PLC-β1 activity after treatment with this growth factor.

ACKNOWLEDGMENTS

The authors thank S. Lach for technical assistance. This work was supported by Associazione Italiana per la Ricerca sul Cancro (AIRC) 1996 and 1997 grants, and from Italian MURST 60% grants to Università di Trieste.

REFERENCES

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PRJ, Reese CB, Cohen P. 1997. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Bα. Curr Biol 7:261–269.

Bahk YY, Lee YH, Lee TG, Seo J, Ryu SH, Suh PG. 1994. Two forms of phospholipase C-β1 generated by alternative splicing. J Biol Chem 273:4465–4469.

Banfic H, Zizak M, Divecha N, Irvine RF. 1993. Nuclear diacylglycerol is increased during cell proliferation in vivo. Biochem J 290:633–636.

Bertagnolo V, Marchisio M, Capitani S, Neri LM. 1997. Intranuclear translocation of phospholipase C β2 during HL-60 myeloid differentiation. Biochem Biophys Res Commun 235:831–837.

Cocco L, Martelli AM, Capitani S, Maraldi NM, Mazzotti G, Barnabei O, Manzoli FA. 1993. Nuclear phosphoinositidase C during growth factor stimulation. Adv Enzyme Regul 33:157–169.

Cocco L, Martelli AM, Gilmour RS. 1994. Inositol lipid cycle and the nucleus. Cell Signal 6:481–485.

Crouch MF. 1991. Growth factor-induced cell division is paralleled by translocation of Gi α to the nucleus. FASEB J 5:200–206.

Divecha N, Banfic H, Irvine RF. 1991. The polyphosphoinositide cycle exists in nuclei of Swiss 3T3 cells under the control of a receptor (for IGF-I) in the plasma membrane, and stimulation of the cycle increases nuclear diacylglycerol and apparently induces translocation of protein kinase C to the nucleus. EMBO J 10:3207–3214.

Divecha N, Rhee S-G, Letcher AJ, Irvine RF. 1993. Phosphoinositide signalling enzymes in rat liver nuclei: Phosphoinositidase C isoform β1 is specifically, but not predominantly, located in the nucleus. Biochem J 289:617–620.

Divecha N, Letcher AJ, Banfic H, Rhee S-G, Irvine RF. 1995. Changes in components of a nuclear inositide cycle during differentiation in murine erythroleukaemia cells. Biochem J 312:63–67.

English JM, Vanderbilt CA, Xu S, Marcus S, Cobb MH. 1995. Isolation of MEK5 and differential expression of alternatively spliced forms. J Biol Chem 270:28897–28902.

Fricker M, Hollinshead M, White N, Vaux D. 1997. Interphase nuclei of many mammalian cell types contain deep, dynamic, tubular membrane-bound invaginations of the nuclear envelope. J Cell Biol 136:531–544.

Giorgetti S, Ballotti R, Kowalski-Chauvel A, Tartare S, Van Obberghen E. 1993. The insulin and insulin-like growth factor-I receptor substrate IRS-1 associates with and activates phosphatidylinositol 3-kinase in vitro. J Biol Chem 268:7358–7364.

Harada R, Smith RM, Smith JA, White M F, Jarett L. 1996. Insulin-induced egr-1 and c-fos expression in 32D cells requires insulin receptor, Shc, and mitogen-activated protein kinase, but not insulin receptor substrate 1 and phosphatidylinositol 3-kinase activation. J Biol Chem 271:30222–30226.

Hansson A, Thorén M. 1995. Activation of MAP-kinase in Swiss 3T3 fibroblasts by insulin-like growth factor I. Growth Regul 5:92–100.

Kim S-J, Kahn CR. 1997. Insulin regulation of mitogen-activated protein kinase (MEK), mitogen-activated protein kinase and casein kinase in the cell nucleus: A possible role in the regulation of gene expression. Biochem J 323:621–627.

Kooijman R, Kappers AC, Rijkers GT. 1995. Insulin-like growth factor induces phosphorylation of immunoreactive insulin receptor substrate and its association with phosphatidylinositol 3-kinase in human thymocytes. J Exp Med 182:593–597.

Kulik G, Klippel A, Weber MJ. 1997. Antiapoptotic signalling by the insulin-like growth factor I receptor, phosphatidylinositol 3-kinase, and Akt. Mol Cell Biol 17:1595–1606.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.

Lee SB, Rhee S-G. 1995. Significance of PIP_2 hydrolysis and regulation of phospholipase C isozymes. Curr Opin Cell Biol 7:183–189.

Lenormand P, Sardet C, Pages G, L'Allemain G, Brunet A, Poyssegur J. 1993. Growth factors induce nuclear translocation of MAP kinase ($p42^{mapk}$ and $p44^{mapk}$) but not of their activator MAP kinase kinase ($p45^{mapkk}$) in fibroblasts. J Cell Biol 122:1079–1088.

Maniotis AJ, Chen CS, Ingber DE. 1997. Demonstration of mechanical corrections between integrins, cytoskeletal filaments, and nucleoplasm that stabilize nuclear structure. Proc Natl Acad Sci USA 94:849–854.

Manzoli L , Billi A M, Rubbini S, Bavelloni A, Faenza I, Gilmour RS, Rhee S-G, Cocco L. 1997. Essential role for nuclear phospholipase C β1 in insulin-like growth factor I-induced mitogenesis. Cancer Res 57:2137–2139.

Marmiroli S, Ognibene A, Bavelloni A, Cinti C, Cocco L, Maraldi NM. 1994. Interleukin 1α stimulates nuclear phospholipase C in human osteosarcoma SaOS-2 cells. J Biol Chem 269:13–16.

Martelli AM, Bareggi R, Cocco L, Manzoli FA. 1996. Stimulation of nuclear polyphosphoinositide synthesis by GTP-γ-S. A potential regulatory role for nuclear GTP-binding proteins. Biochem Biophys Res Commun 218:182–186.

Martelli AM, Billi AM, Gilmour RS, Neri LM, Manzoli L, Ognibene A, Cocco L. 1994. Phosphoinositide signalling in nuclei of Friend cells: phospholipase C β down regulation is related to cell differentiation. Cancer Res 54:2536–2540.

Martelli AM, Gilmour RS, Bertagnolo V, Neri LM, Manzoli L, Cocco L. 1992. Nuclear localisation and signalling activity of phosphoinositidase C β in Swiss 3T3 cells. Nature 358:242–245.

Meier R, Alessi DR, Cron P, Andjelkovic M, Hemmings BA. 1997. Mitogenic activation, phosphorylation, and nuclear translocation of protein kinase Bβ. J Biol Chem 272: 30491–30497.

Morishima-Kawashima M, Kosik KS. 1996. The pool of MAP kinase associated with microtubules is small but constitutively active. Mol Biol Cell 7:893–905.

Neri LM, Ricci D, Carini C, Marchisio M, Capitani S, Bertagnolo V. 1997. Changes of nuclear PI-PLC γ1 during rat liver regeneration. Cell Signal 9:353–362.

O'Connor R, Kauffmann-Zeh A, Liu Y, Lehar S, Evan GI, Baserga R, Blattler WA. 1997. Identification of domains of insulin-like growth factor I receptor that are required for protection from apoptosis. Mol Cell Biol 17:427–435.

Parrizas M, Saltiel AR, Leroith D. 1997. Insulin-like growth factor I inhibits apoptosis using the phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways. J Biol Chem 272:154–161.

Pienta KJ, Coffey DS. 1992. Nuclear-cytoskeletal interactions: evidence for physical connections between the nucleus and cell periphery and their alteration by transformation. J Cell Biochem 49:357–365.

Rhee SG, Bae YS. 1997. Regulation of phosphoinositide-specific phospholipase C isozymes. J Biol Chem 272: 15045–15048.

Rubbini S, Cocco L, Manzoli L, Lutterman J, Billi AM, Matteucci A, Wirtz KWA. 1997. Phosphoinositide signalling in nuclei of Friend cells: DMSO-induced differentiation reduces the association of phosphatidylinositol-transfer protein with the nucleus. Biochem Biophys Res Commun 230:302–305.

Ryu SH, Kim U-H, Wahl MI, Brown AB, Carpenter G, Huang K-P, Rhee SG. 1990. Feedback regulation of phospholipase C-β by protein kinase C. J Biol Chem 265: 17941–17945.

Saffitz JE, Nash JA, Green KG, Luke RA, Ransnas LS, Insel PA. 1994. Immunoelectron microscopic identification of cytoplasmic and nuclear Gs α in S49 lymphoma cells. FASEB J 8:252–258.

Schmalz D, Kalkbrenner F, Hucho F, Buchner K. 1996. Transport of protein kinase C α into the nucleus requires intact cytoskeleton while the transport of a protein containing a canonical nuclear localization signal does not. J Cell Sci 109:2401–2406.

Scrimgeour AG, Blakesley VA, Stannard BS, LeRoith D. 1997. Mitogen-activated protein kinase and phosphatidylinositol 3-kinase pathways are not sufficient for insulin-like growth factor I-induced mitogenesis and tumorigenesis. Endocrinology 138:2552–2558.

Sun B, Murray NR, Fields AP. 1997. A role for phosphatidylinositol-specific phospholipase C in the G2/M phase transition. J Biol Chem 272:26313–26317.

Takahashi Y, Tobe K, Kadowaki H, Katsumata D, Fukushima Y, Yazaki Y, Akanuma Y, Kadowaki T. 1997. Roles of insulin receptor substrate-1 and Sch on insulin-like growth factor I receptor signaling in early passages of cultured human fibroblasts. Endocrinology 138:741–750.

Valentinis B, Morrione A, Taylor SJ, Baserga R. 1997. Insulin-like growth factor I receptor signaling in transformation by src oncogenes. Mol Cell Biol 17:3744–3754.

York JD, Majerus PW. 1994. Nuclear phosphatidylinositols decrease during S-phase of the cell cycle in HeLa cells. J Biol Chem 269:7847–7850.

York JD, Saffitz E, Majerus PW. 1994. Inositol polyphosphate 1-phosphatase is present in the nucleus and inhibits DNA synthesis. J Biol Chem 269:19992–19999.

Zauli G, Bassini A, Vitale M, Gibellini D, Celeghini C, Caramelli E, Pierpaoli S, Guidotti L, Capitani S. 1997. Thrombopoietin enhances the aIIbγ3-dependent adhesion of megakaryocytic cells to fibrinogen or fibronectin through PI 3 kinase. Blood 89:883–895.

Zini N, Martelli AM, Cocco L, Manzoli FA, Maraldi NM. 1993. Phosphoinositidase C isoforms are specifically localized in the nuclear matrix and cytoskeleton of Swiss 3T3 cells. Exp Cell Res 208:257–269.

Zini N, Ognibene A, Marmiroli S, Bavelloni A, Maltarello MC, Faenza I, Valmori A, Maraldi NM. 1995. The intranuclear amount of phospholipase C β1 decreases following cell differentiation in Friend cells, whereas γ1 isoform is not affected. Eur J Cell Biol 68:25–34.