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ORIGINAL ARTICLE

The kinase c-Src and the phosphatase TC45 coordinately regulate c-Fos tyrosine phosphorylation and c-Fos phospholipid synthesis activation capacity

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Our previous work showed that in T98G cells, a human glioblastoma multiforme-derived cell line, the association of c-Fos to the endoplasmic reticulum (ER) and consequently, the capacity of c-Fos to activate phospholipid synthesis, is regulated by the phosphorylation state of tyrosine (tyr) residues #10 and #30 of c-Fos. The small amount of c-Fos present in quiescent cells is tyr-phosphorylated, is dissociated from the ER membranes and does not activate phospholipid synthesis. However, on induction of the cell to re-enter growth, c-Fos expression is rapidly induced, it is found dephosphorylated, associated to ER membranes and activating phospholipid synthesis (Portal *et al.*, 2007). Herein, using *in vivo* and *in vitro* experimental strategies, we show that the kinase c-Src is capable of phosphorylating tyr residues of c-Fos whereas the phosphatase TC45 T-cell protein-tyr phosphatase (TC-PTP) dephosphorylates them, thus enabling c-Fos/ER association and activation of phospholipid synthesis. Results also suggest that the regulation of the phosphorylation/dephosphorylation cycle of c-Fos occurs at the TC-PTP level: induction of cells to re-enter growth promotes the translocation of TC45 from a nuclear to a cytoplasmic location concomitant with its activation. Activated TC45 in its turn promotes dephosphorylation of pre-formed c-Fos, enabling cells to rapidly activate phospholipid synthesis to respond to its growth demands.
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Introduction

The expression of the proto-oncoprotein c-Fos is very tightly regulated responding rapidly and transiently to a plethora of stimuli (Angel and Karin, 1991; Morgan and Curran, 1995; Caputto and Guido, 2000). c-Fos heterodimerizes with proteins of the *jun* family to form some of the many AP-1 transcription factors that regulate the expression of target genes among which are genes involved in the initiation of DNA synthesis as a response to growth factors (Kouzarides and Ziff, 1989; Angel and Karin, 1991). A precise molecular understanding of the participation of AP-1 in complex processes such as proliferation and growth has not yet been achieved in spite of having been described >20 years ago and of the many studies directed toward establishing the target genes of the different AP-1 dimers.

Results from our laboratory have disclosed that in addition to its nuclear activity, c-Fos exerts a cytoplasmic activity: it associates to components of the endoplasmic reticulum (ER) where the main cellular production of phospholipids takes place, and activates their synthesis. c-Fos–ER association and consequently the capacity of c-Fos to activate phospholipid synthesis, is regulated by the phosphorylation state of tyrosine (tyr) residues #10 and #30 of c-Fos: quiescent cells contain small amounts of c-Fos, which is tyr-phosphorylated and is neither associated to the ER membranes nor does it activate phospholipid synthesis. However, on induction of cells to re-enter growth, c-Fos expression rapidly increases, c-Fos is dephosphorylated and is found associated to the ER membranes and capable of activating phospholipid synthesis (Portal *et al.*, 2007).

The c-Fos-dependent activation of lipid synthesis has been implicated in cell differentiation, proliferation and growth. This lipid-activating capacity of c-Fos has been observed *in vivo* in events related to the neuronal activity of chick retina ganglion and photoreceptor cells (Guido *et al.*, 1996; Bussolino *et al.*, 1998; de Arriba Zerpa *et al.*, 1999) and also *in culture* in NIH3T3 cells induced to re-enter growth (Bussolino *et al.*, 2001) and in PC12 cells induced to differentiate (Gil *et al.*, 2004; Crespo *et al.*, 2008). The biological relevance of c-Fos-dependent lipid synthesis activation was underscored in human brain malignant tumor cells that express abundant c-Fos, contrasting with a non-detectable expression of this protein in normal human brain specimens. Furthermore, in these tumors, c-Fos is associated to ER components and activates phospholipid synthesis as

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compared with normal specimens. In accordance, in an animal model of Neurofibromatosis Type I, the NPcis mouse that spontaneously develops nervous system tumors, tumor development depends on c-Fos expression: no tumors develop in NPcis mice KO for c-Fos contrasting with a tumor burden >70% in their NPcis littermate animals, with no significant modifications in the AP-1 content (Silvestre *et al*., 2010). In line with these observations is our finding that in T98G cells, impairing tyr-dephosphorylation before inducing cells to re-enter growth results in accumulation of phosphorylated c-Fos, no c-Fos-dependent phospholipid synthesis activation and inhibition of cell proliferation (Portal *et al*., 2007).

The reversible phosphorylation state of protein tyr residues catalyzed by the coordinated action of protein kinases and protein-tyr phosphatases (PTPs) is crucial for controlling proliferation, differentiation, survival and metabolism (Hunter, 2000), all processes in which c-Fos participates (Angel and Karin, 1991; Gil *et al*., 2004; Silvestre *et al*., 2010). The aim of this work was to identify the kinases(s) and phosphatase(s) responsible for regulating c-Fos tyr-phosphorylation, c-Fos/ER association and consequently, c-Fos-dependent phospholipid synthesis activation. We found that cytoplasmic c-Src (Oppermann *et al*., 1979) phosphorylates c-Fos and that the 45 Kd-variant of Human T-cell protein-tyr phosphatase (TC-PTP) (Cool *et al*., 1989) dephosphorylates c-Fos tyr-residues #10 and #30. Results indicate that activation of TC-PTP on stimulation of cells to re-enter growth promotes c-Fos dephosphorylation, enabling pre-formed c-Fos to associate to the ER membranes and rapidly activate lipid synthesis in response to the growth stimulus.

Results

Recombinant c-Src phosphorylates c-Fos in vitro

An *in silico* study using the Human Protein Reference Database to identify possible kinases(s) responsible for phosphorylating c-Fos tyr residues shed two putative kinases: the epidermal growth factor receptor and c-Src. As c-Fos has been found in the cell nucleus (Morgan and Curran, 1995) and in the vicinity of or associated to ER membrane components (Bussolino *et al*., 2001; Gil *et al*., 2004; Silvestre *et al*., 2010) and c-Src has been localized to the perinuclear region of the cell (Frame, 2002), we investigated if c-Src is a kinase that recognizes c-Fos as one of its substrates to phosphorylate it on tyr residues.

c-Src was assayed to determine its capacity to phosphorylate c-Fos *in vitro* (Cooper *et al*., 1984) by incubation of recombinant c-Src with c-Fos. Incubates were immunoblotted with anti-P-Tyr (P-Tyr) antibody. Incubation of c-Fos with c-Src results in the tyr phosphorylation of c-Fos (Figure 1). In parallel, we examined if c-Src phosphorylates the c-Fos mutant Y10/30F in which tyr residues #10 and #30 are substituted by non-phosphorylatable phenylalanine. Phosphorylation of Y10/30F was negligible (Figure 1) indicating that

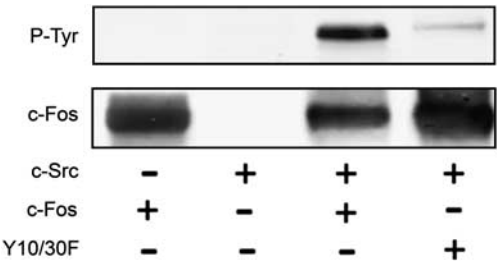

Figure 1 c-Src phosphorylates c-Fos *in vitro*. c-Fos or of the non-phosphorylatable c-Fos mutant Y10/30F were incubated with recombinant c-Src for 30 min at 30°C. Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by WB using anti-P-tyr antibody (upper panel); then membranes were stripped and labelled with anti-c-Fos antibody (lower panel) as a loading control.

in vitro, c-Fos phosphorylation by c-Src occurs on the two tyr residues previously found phosphorylated *in vivo* (Portal *et al*., 2007).

Endogenous c-Src phosphorylates c-Fos in vitro

To determine if endogenous c-Src is also capable of phosphorylating c-Fos *in vitro,* T98G cell lysates were subjected to immunoprecipitation (IP) with a c-Src antibody and IP c-Src activity was determined *in vitro* with recombinant c-Fos as substrate. To gain information on the possible regulatory mechanism imposed by cells on c-Fos phosphorylation, the activity of endogenous c-Src from quiescent cells was examined to see if an activation or inhibition of c-Src activity is promoted by the growth conditions.

As observed with recombinant c-Src in Figure 1, endogenous c-Src also phosphorylates c-Fos as was evidenced after 5 min of incubation (Figure 2a, upper panel). Noticeably, no differences were found between c-Src activity in quiescent (zero time) and stimulated cells (+fetal bovine serum (FBS) for 7.5 or 60 min) at any incubation time examined (Figure 2a, upper panel). The middle and lower panels of Figure 2a are loading controls of c-Fos and c-Src, respectively, assayed in the upper panel.

Apart from demonstrating that c-Src is capable of phosphorylating c-Fos *in vitro*, results indicate that its activity toward c-Fos is the same independently of the cells' growth conditions. To discard that the apparent lack of regulation of c-Src-phosphorylation of c-Fos was due to some modification in endogenous c-Src that could turn it insensitive to the growth conditions, the c-Src phosphorylating activity of another well-described c-Src substrate, enolase (Hu *et al*., 2007), was determined. The activity of IP c-Src on enolase is clearly activated on inducting cells to re-enter growth (Figure 2b, first row). Taken together, these results suggest that the regulation of the phosphorylation state of c-Fos is not associated with cell growth-dependent fluctuations in c-Src kinase activity.

c-Src and c-Fos colocalize in T98G cells

Although the experiments described above indicate that recombinant (Figure 1) and endogenous (Figure 2) c-Src are able to phosphorylate c-Fos *in vitro*, this may not be

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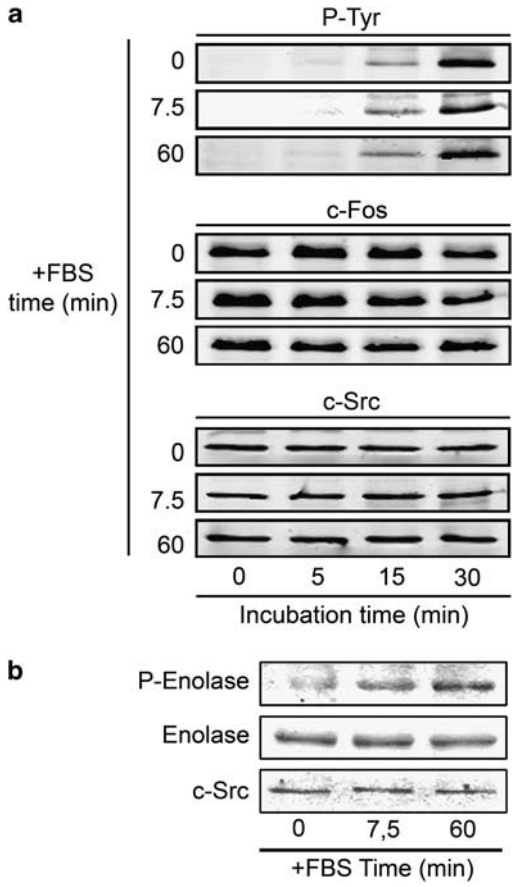

Figure 2 Endogenous c-Src phosphorylates c-Fos. (**a**) Endogenous c-Src IP from quiescent cells (0 min) or from cells induced to grow (+FBS) for 7.5 or 60 min were incubated with recombinant c-Fos for the times indicated; P-c-Fos obtained was determined by WB using anti-P-tyr antibody (upper panel). Membranes were then stripped and exposed to anti c-Fos antibody (middle panel) and to anti-c-Src antibody (lower panel) as a loading control. (**b**) Activity of the IP c-Src determined in (**a**) was assayed using enolase as substrate; in all cases incubations were for 15 min. Note that phosphorylation capacity of c-Src on enolase increases with increasing time of cell growth.

relevant if both proteins localize in different sub-cellular locations. To determine if c-Fos and c-Src co-distribute at the sub-cellular level, quiescent and growing T98G cells were immunostained for endogenous c-Fos, c-Src and the ER marker GRP78. Figure 3 shows the expected induction of c-Fos expression in cells induced to grow by feeding with FBS (+FBS) as compared with quiescent (–FBS) cells (compare Figures 3a and i). In growing cells, the immuno-staining patterns of c-Fos, c-Src and the ER were similar (compare Figures 3i–k) with evident colocalization of the three markers (see merged images in Figure 3l). Colocalization was much less evident in quiescent cells (Figures 3a–c and merged images in d) probably due both to the low levels of c-Fos in the cells (Figure 3a) and to the fact that after culturing cells for 48 h –FBS to attain quiescence, most of the c-Fos contained by the cells is already phosphorylated and dissociated from the ER membranes (Portal *et al*., 2007).

c-Fos phosphorylated by c-Src no longer activates phospholipid synthesis

It was considered of interest to know if phosphorylation of c-Fos by c-Src *in vitro* modifies its capacity to activate phospholipid synthesis. For this, we assayed the capacity to activate phospholipid synthesis of c-Src-phosphorylated c-Fos (P-c-Fos), its non-phosphorylatable mutant Y10/30F and of Y10/30E, a phosphomimetic version of c-Fos in which glutamic acid residues substitute for tyr residues #10 and #30. c-Fos and the Y10/30F mutant were first subjected to the c-Src phosphorylation procedure described under Figure 1. Then, these proteins were assayed to determine their *in vitro* capacity to activate phospholipid synthesis as described under Figure 4. Y10/30F not incubated with c-Src and c-Src incubated without any added substrates were run as controls.

Figure 4 shows that neither recombinant c-Fos phosphorylated *in vitro* by c-Src or the phosphomimetic mutant Y10/30E increase phospholipid synthesis above control levels. By contrast, non-phosphorylatable Y10/30F, treated or not with c-Src, has the same capacity to activate phospholipid synthesis as c-Fos. c-Src alone does not modify phospholipid synthesis. These results indicate that for c-Fos to activate phospholipid synthesis, both tyr residues #10 and #30 must be devoid of an acidic residue such as that conferred by the phosphate residues or, in Y10/30E, by glutamic acid, an acidic amino acid.

c-Fos- and Y10/30F- but not Y10/30E-expressing cells show activated phospholipid synthesis and progress through the cell cycle

To determine if c-Fos activation of phospholipid synthesis is relevant for cell proliferation, quiescent T98G cells were transfected to express c-Fos or Y10/30E or Y10/30F or were cultured in the presence of sense (SO) or anti-sense (ASO) c-Fos mRNA oligonucleotide. Figure 5 shows that phospholipid synthesis is activated in cells cultured 30 h +FBS to induce c-Fos expression (black columns 3 and 4). This was also so in those transfected to express c-Fos or Y10/30F and primed 9 h +FBS (black columns 7 and 8). By contrast, cells transfected to express Y10/30E and primed 9 h +FBS showed levels of phospholipid synthesis comparable to those cultured the 30 h –FBS (compare black columns 6 with 1) or to non-transfected cells primed 9 h +FBS (black column 2). Cells cultured 30 h +FBS, +ASO, showed no phospholipid synthesis activation (column 5).

Cell proliferation strictly correlates with the condition of activated phospholipid synthesis. Figure 5 shows that cells primed 9 h +FBS and then cultured –FBS to complete 30 h proliferate only if phospholipid synthesis is activated, that is, in cells transfected to express c-Fos (column 7) or Y10/30F (column 8). Cells in which c-Fos expression is impaired with ASO (column 5) or express the phosphomimetic version of c-Fos, Y10/30E (column 6) neither activate phospholipid synthesis (black columns) or proliferate (grey columns).

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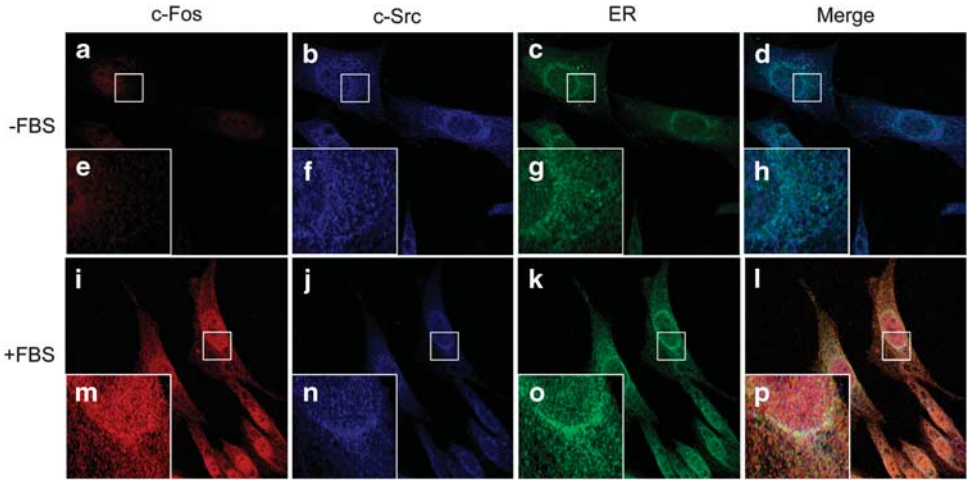

Figure 3 c-Fos and c-Src colocalize in T98G cells. Quiescent cells (grown –FBS for 48 h, top row) and growing cells (+ FBS 1 h before harvesting; bottom row) were immunostained for c-Fos (**a**, **i**), for c-Src (**b**, **j**) and for the ER marker GRP78 (**c**, **k**) and examined by confocal microscopy at 100 × . To the right (**d**, **l**) is the merged image of the three; (**e**–**h**) and (**m**–**p**) are magnified images of each inset.

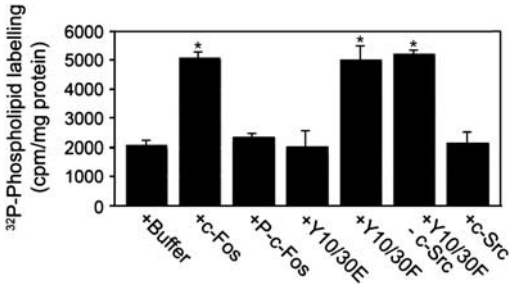

Figure 4 c-Fos phosphorylated by c-Src does not activate phospholipid synthesis. The capacity to activate phospholipid synthesis of recombinant c-Fos, c-Fos phosphorylated by purified c-Src (P-c-Fos), the phosphomimetic Y10/30E mutant of c-Fos and the non-phosphorylatable mutant of c-Fos Y10/30F was examined as described previously (Gil *et al*., 2004). Briefly, ^{32}P-phospholipid labelling capacity of quiescent cells was determined *in vitro* by the addition of 100 μg of quiescent cell homogenate to the assay medium containing in a final vol of 80 μl, 140 mM NaCl, 4.5 mM KCl, 0.5 mM $MgCl_2$, 5.6 mM glucose, 64 mM HEPES buffer pH 7.4 and 3 μC of $^{[32]}$P-ATP (spec.act. 3000 Ci/mmol; PerkinElmer Life Sciences) plus 1ngr of either recombinant c-Fos protein, P-c-Fos, Y10/30F, or Y10/30E/μg of homogenate protein. Incubations were for 90 min at 37 °C. ^{32}P-phospholipid quantification was performed as described previously (Guido and Caputto, 1999). Results expressed as c.p.m. of ^{32}P incorporated into phospholipids/mg of protein are the mean ± s.d. of three experiments performed in triplicate; $*P<0.002$ with respect to control (buffer) as determined by Student's two-tailed *t*-test. Y10/30F not incubated with c-Src and c-Src incubated without any added substrates were run as controls. Note that the presence of c-Src in the assays did not modify phospholipid synthesis.

c-Src accounts for most of the endogenous kinase activity that phosphorylates c-Fos in NIH3T3 cells in culture

To know if endogenous c-Src activity is relevant for endogenous c-Fos phosphorylation, phosphorylation of c-Fos in quiescent NIH3T3 cells knock out for c-Src (Src–/–) or for c-Src plus two other members of the Src family, c-Yes and c-Fyn (SYF–/–) was examined and compared with c-Fos phosphorylation in wt NIH3T3 fibroblasts.

Negligible levels of phosphorylated c-Fos were recovered both from Src–/– and SYF–/– cells as compared with wt cells (Figure 6a, top row). Figure 6a (bottom row) shows a c-Fos loading control. The capacity of these cells to synthesize phospholipids was also examined. Figure 6b shows that in spite that cells are quiescent, phospholipid synthesis capacity is significantly higher in cells that do not phosphorylate c-Fos, that is, in Src–/– and SYF–/– cells as compared with wt fibroblasts, indicating that c-Src is quantitatively relevant for the phosphorylation of endogenous c-Fos in quiescent cells.

TC-PTP dephosphorylates c-Fos

The Human Protein Reference Database was used to identify putative phosphatase(s) that may dephosphorylate c-Fos. This *in silico* analysis yielded the family of TC-PTP's as putative c-Fos phosphatases. Two forms of human TC-PTP with identical catalytic domains but different non-catalytic C-terminal domains have been described: a 48-kDa form (TC48) that is targeted to the ER by a stretch of hydrophobic residues at the extreme C terminus, and a 45-kDa variant (TC45) that lacks the hydrophobic C-terminal tail and is targeted to the nucleus by an atypical bipartite nuclear localization sequence (Lorenzen *et al*., 1995; Tiganis *et al*., 1997; Ibarra-Sanchez *et al*., 2000). TC45 exits the nucleus in response to specific stimuli to dephosphorylate distinct substrates thus regulating specific signalling pathways (Tiganis *et al*., 1998, 1999; Tiganis, 2002).

T98G cells were examined for the presence of transcripts of the TC45 and TC48 isoforms of TC-PTP's by reverse transcriptase–PCR. The analysis was carried out in quiescent cells and in cells stimulated with FBS for 5 or 60 min, times at which cells contain predominantly dephosphorylated c-Fos (Portal *et al*., 2007). Figure 7a (left panel) shows that only the TC45 mRNA isoform is present in these cells and no apparent

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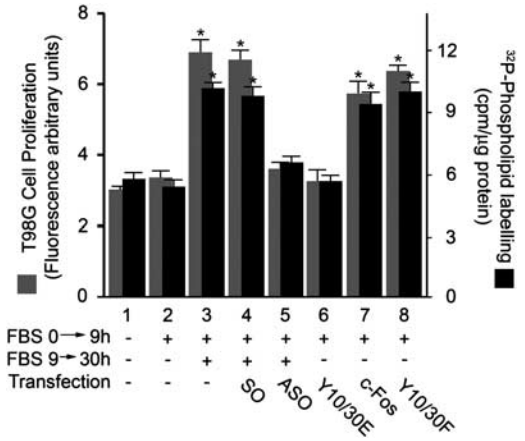

Figure 5 c-Fos- and Y10/30F- but not Y10/30E-expressing cells show activated phospholipid synthesis and progress through the cell cycle. Quiescent T98G cells non-transfected (columns 1–3) or transfected to express c-Fos or the indicated mutant versions (columns 6–8) were examined for their capacity to stimulate labelling of phospholipids *in culture* from ^{32}P (black columns) and also for cell proliferation (grey columns). Cells were primed to proliferate by feeding with FBS during 9 h, time at which medium was changed to fresh medium –FBS (columns 2, 6–8) or + FBS (columns 3–5) and + ASO (column 5) or + SO (column 4), added to the medium 3 h before initiating cell priming by FBS addition. Column 1 corresponds to cells incubated the 30-h –FBS. Phospholipid labelling was determined as described previously (Guido and Caputto, 1990) after pulsing cells with ^{32}P-orthophosphate during 2 h before harvesting at 30 h. Results are the mean c.p.m. incorporated into phospholipids/µg protein ± s.d. of three experiments performed in triplicate; $*P<0.002$ with respect to column 1 as determined by Student's two-tailed *t*-test. Proliferation was determined using the CyQUANT NF Cell Proliferation Assay Kit (Invitrogen) according to manufacturer's protocol. Results expressed as the mean arbitrary fluorescence intensity units ± s.d. of three experiments performed in sixtuplicate; $*P<0.001$ with respect to column 1 as determined by Student's two-tailed *t*-test. Note that proliferation of primed cells occurs only in cells with activated phospholipid synthesis.

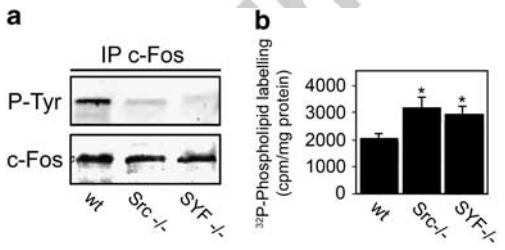

Figure 6 c-Src accounts for most of the endogenous kinase activity that phosphorylates c-Fos in NIH3T3 cells. (**a**) NIH3T3 wt fibroblasts, and fibroblasts KO for c-Src (c-Src–/–) and KO for c-Src plus c-Yes plus c-Fyn (SYF–/–) were examined for the phosphorylation status of endogenous c-Fos. Cell lysates were IP with anti c-Fos antibody; IP were subjected to WB with anti-P-Tyr antibody (upper panel). Stripped blots were then immunostained with anti c-Fos antibody as a loading control (lower panel). Results are from one representative experiment of two performed, both of which gave essentially the same results. (**b**) *In vitro* phospholipid synthesis was determined using cell homogenates from the quiescent cells shown in (**a**). Results are the mean ± s.d. of three experiments performed in triplicate; $*P<0.002$ with respect to wt as determined by Student's two-tailed *t*-test.

induction of its expression is promoted when cells are stimulated to grow with FBS for 5 or 60 min as also evidenced in the quantification to the right. No TC48-mRNA could be detected either in quiescent or growing cells at any time examined (Figure 7a, middle panel). Consequently, the studies that followed were focused on determining the capacity of TC45 to dephosphorylate c-Fos *in vitro* and *in vivo*.

To examine the capacity of purified TC45 to dephosphorylate P-c-Fos *in vitro*, c-Fos was first phosphorylated by incubation in the presence of recombinant c-Src as described in Figure 1 and incubates subjected to WB using anti-P-Tyr antibody to control c-Fos tyr-phosphorylation (Figure 7b, left panel, first row). Membranes were stripped, washed thoroughly with TBS and incubated in the presence (+TC45) or the absence (–TC45) of recombinant TC45. Membranes were then incubated with anti-P-Tyr antibody to examine the phosphorylation state of c-Fos. It was found that TC45 significantly dephosphorylates c-Fos as compared with control assays run in parallel in the absence of TC45 (Figure 7b, left panel, compare lanes 3 and 4 and quantification to the right).

The capacity of c-Fos to activate phospholipid synthesis according to its phosphorylated state was also examined. As expected considering the results of Figure 4, P-c-Fos has no lipid synthesis-activating capacity, but is restored on treatment with TC45 (Figure 7c, compare second and forth columns). A control was run to discard that the presence of TC45 in the assays was modifying phospholipid labelling rates (Figure 7c, last column).

c-Fos and TC45 co-IP

Several cellular substrates of TC45 have been identified including the epidermal growth factor receptor, the adaptor protein p52Shc (Tiganis *et al*., 1998, 1999; Klingler-Hoffmann *et al*., 2001), the Janus family of kinases 1 and 3 (Simoncic *et al*., 2002) and the signal transducer and transcription activator, STAT1 (Aoki and Matsuda, 2002; Yamamoto *et al*., 2002). To the best of our knowledge, c-Fos has not yet been considered a possible substrate of TC45. To confirm that TC45 is indeed capable of interacting with c-Fos, cells were double transfected with HA-tagged c-Fos and myc-tagged TC45 or with a point mutated substrate-trapping version of TC45, D182A (Tiganis *et al*., 1998), also myc-tagged. D182A has an Asp/Ala mutation about 30 amino acids N-terminal of the catalytic HCSAG motif (Wälchli *et al*., 2000). Like its *wt* counterpart TC45, D182A forms an enzyme–substrate intermediate involving the interaction of the conserved phosphatase cysteine with the tyr phosphate of the substrate forming a stable complex that, at difference with TC45, is unable to release the dephosphorylated substrate.

Cells were double transfected 48 h before harvesting and immunocomplex formation between TC45 or D182A with c-Fos was examined both in quiescent and in cells induced to grow by addition of FBS for 15 min. Figure 8A shows that c-Fos co-IP with both

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TC45 and D182A although more immunocomplex formation was observed with D182A. Comparison of immunocomplex formation between growing and quiescent cells evidenced that the amount of c-Fos/TC45 and c-Fos/D182A immunocomplex formation in growing cells was higher than in quiescent ones (Figure 8A, top row). Quantification of c-Fos/PTP immunocomplex formation shown in the right panel of Figure 8A evidenced a $\sim 2\times$ increase in growing with respect to quiescent cells, both with TC45 and with D182A. The bottom panel of Figure 8A is a c-myc-PTP and HA-c-Fos loading control.

Cellular localization of HA-c-Fos and green fluorescent protein-tagged D182A and of the ER marker GRP78 was examined by immunofluorescence in quiescent and growing, co-transfected cells. Figure 8B shows the expected translocation of D182A from a preponderantly nuclear localization in quiescent cells (panel c) to a cytoplasmic one in cells cultured + FBS for 15 min (panel d) (Galic *et al*., 2003). Importantly, translocated cytoplasmic D182A shows clear colocalization sites with c-Fos in + FBS cells (inset to panel d); c-Fos also colocalizes with the ER marker (inset to panel f). Figure 8B (panel h) shows colocalization sites of the three markers examined in + FBS cells.

TC45 activity is substantial for dephosphorylating c-Fos

This activity is regulated according to the physiological state of the cell. To determine the contribution of TC45 to the total phosphatase activity in T98G cells that may dephosphorylate c-Fos, TC45 activity was blocked by transfecting cells with micro-RNA against TC45 (miR-TC45) 48 h before cell harvesting and the presence of P-c-Fos determined. As a control, a plasmid that does not affect TC45 expression (miR-Neg) was transfected in parallel. miR-TC45 was effective in impairing TC45 expression as compared with control cells grown in the absence of miR-TC45 (–miR-TC45 cells) (Figure 9a, lanes 1 and 2) or to those treated with miR-Neg (Figure 9a, last lane). The right panel of Figure 9a is the quantification of the left panel.

Next, the cellular content of phosphorylated c-Fos in quiescent (–FBS) and growing (+ FBS for 15 min) cells cultured 48 h + miR-TC45 or + miR-Neg was examined. P-c-Fos was clearly detectable in cells transfected with miR-TC45 and cultured both in the presence (+ FBS) and the absence (–FBS) of FBS (Figure 9b,

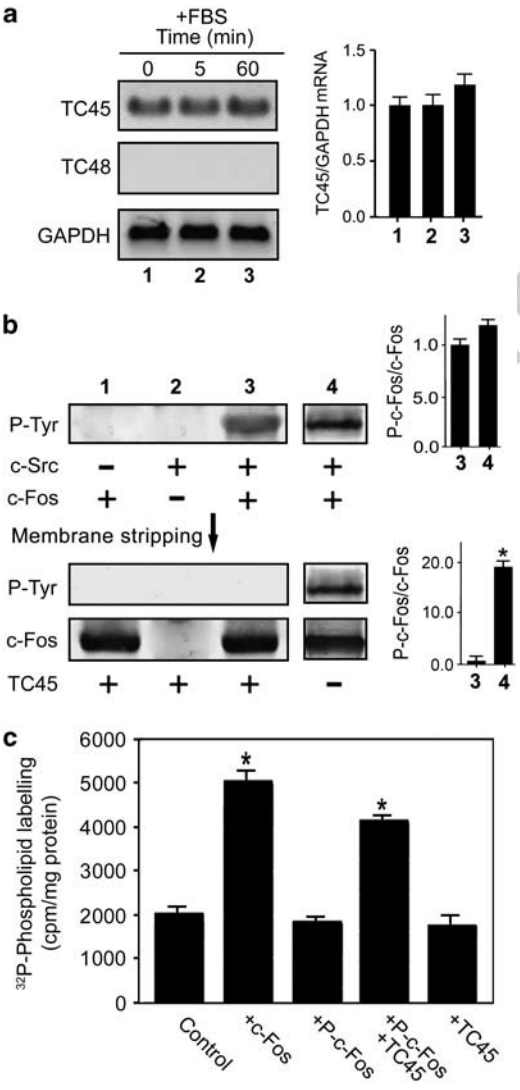

Figure 7 TC45 but not TC48 phosphatase is present in T98G cells; TC45 dephosphorylates c-Fos. (**a**) RT–PCR determination of TC45 (upper panel) or TC48 (middle panel) mRNA in quiescent cells (0 min, lane 1) and in cells induced to grow by feeding of FBS for 5 (lane 2) or 60 min (lane 3). Only TC45 mRNA was found in these cells. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA (bottom panel) was used as a loading control. Quantification of TC45 mRNA is given in the right panel as the ratio of the optical density units of TC45 mRNA/GAPDH mRNA ± s.d. of three experiments performed in triplicate. The ratio in lane 1 was arbitrarily taken as one. No significant differences were found between the values obtained after 0, 5 or 60 min of FBS treatment as determined by Student's two-tailed *t*-test. (**b**) Recombinant c-Fos was incubated in the presence (+ c-Src, lanes 3 and 4) or the absence (−Src, lane 1) of recombinant c-Src, A control incubate or c-Src incubated incubated in the same conditions but in the absence of c-Fos was run in parallel (lane 2). Incubates were then subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and WB using anti-P-Tyr antibody (Figure 7b, upper panel). Membranes were then stripped, washed thoroughly, incubated in the presence (+ TC45, lanes 1–3) or the absence (−TC45, lane 4) of recombinant TC45 and re-submitted to WB with anti-P-Tyr antibody (middle panel of Figure 7b). Remnant c-Fos in the membrane is shown on the lower panel of Figure 7b whereas lane 4 shows P-c-Fos and c-Fos content at different steps of the experimental procedure. To the right is the quantification of lanes 3 and 4 of the corresponding WB shown to the left. Results are the mean ratio of the optical density units of P-c-Fos/c-Fos ± s.d. of three experiments performed in triplicate; $*P<0.001$ as determined by Student's two-tailed *t*-test. The ratio in lane 3 was arbitrarily taken as one. (**c**) Phosphorylated and TC45 dephosphorylated c-Fos obtained as in (**b**) were assayed *in vitro* for their capacity to activate phospholipid synthesis. A control incubate of TC45 without c-Fos (+ TC45) was included in the assay. Results expressed as c.p.m./mg of protein of ^{32}P incorporated into phospholipids are the mean ± s.d. of two experiments performed in triplicate; $*P<0.002$ with respect to the control as determined by Student's two-tailed *t*-test.

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lanes 3 and 4, top row) whereas in miR-Neg-transfected cells, the remnant P-c-Fos found in +FBS cells (lane 2) was at the limit of detection (compare lane 2 with 1 or 3). The right panel shows the quantification of the left panel. These results suggest that when quiescent cells are induced to grow, concomitant to the induction of c-Fos expression, an activation of P-c-Fos dephosphorylation by cellular TC45 occurs thus enabling a rapid activation of the metabolic response of the cell. In the absence of TC45 activity, P-c-Fos present in quiescent cells is not dephosphorylated when cells are induced to re-enter growth (Figure 9b, lane 4). Loading controls for c-Fos are shown in the bottom row of Figure 9b.

In vitro phospholipid labelling capacity of cells treated as in Figure 9b was also examined but in the presence of cycloheximide to avoid induction of c-Fos expression on FBS addition. As expected, activated phospholipid synthesis was found only in homogenates containing dephosphorylated c-Fos (lane 2, Figure 9c).

To confirm that mitogens that induce c-Fos expression also activate TC45 activity, we determined the activity of TC45 present in lysates prepared from quiescent cells and compared it with that from cells induced to grow for the last 15 min before harvesting. TC45 from +FBS cells (Figure 9d, top row, lane 2) was found more active to dephosphorylate P-c-Fos *in vitro* than that from quiescent (–FBS) cells (Figure 9d, top row, lane 1). To the left is the quantification of the remnant P-c-Fos with respect to c-Fos after treatment with TC45 from –FBS and +FBS cells. Loading controls for c-Fos and TC45 are shown on middle and bottom rows of the left panel of Figure 9d.

Discussion

It seems reasonable to expect a rigorous control on the capacity of c-Fos to activate phospholipid synthesis because of the importance it has on the metabolic outcome of a cell: c-Fos can be activating or not the synthesis of lipids and consequently can increase or not the rate of membrane biogenesis. This control may be achieved either by controlling the expression of this protein or by controlling its capacity to activate lipid synthesis. The induction of c-Fos expression in response to extracellular stimuli is a well-recognized characteristic of c-Fos and has been extensively documented (reviewed in Angel and Karin, 1991; Morgan and Curran, 1995; Caputto and Guido, 2000). As the cellular responses to extracellular stimuli must be rapid, the *de novo* synthesis of proteins, in this case of c-Fos, appears at first sight as an event too slow to elicit a rapid response in the cell.

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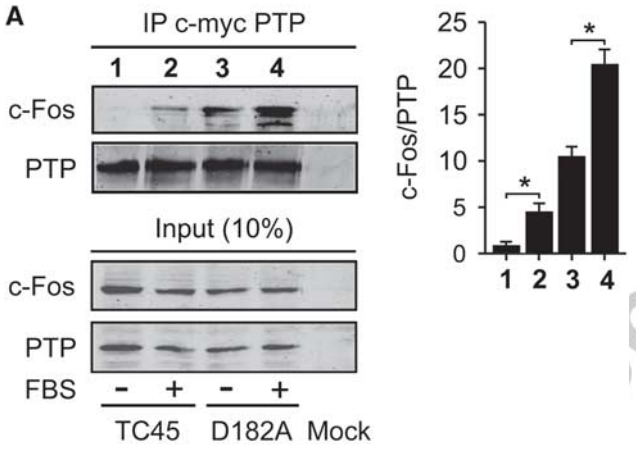

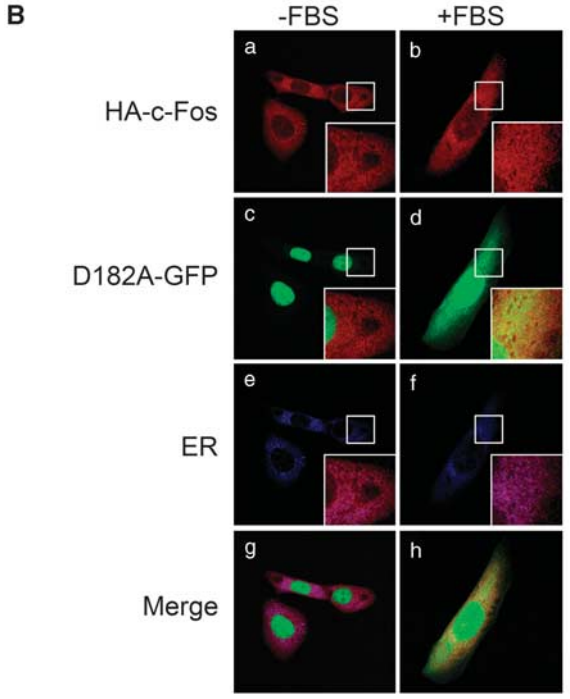

Figure 8 c-Fos interacts with TC45. (**A**) Lysates prepared from quiescent (–FBS) or growing cells (+FBS during 15 min) double transfectants for HA-c-Fos and myc-TC45 or HA-c-Fos and myc-D182A, were IP with anti-myc antibody. Immunocomplexes were analyzed after sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and WB using anti-HA (upper row) and anti-myc antibody (lower row). The expression levels of the transfected proteins (10% of that used for IP) were visualized using anti-c-myc or anti c-HA antibodies, as indicated. To the right is the quantification of lanes 1–4 of the WB shown to the left expressed as the mean of the optical density ratio c-Fos/PTP (TC45 or D182A) of the upper panel ± s.d. of three experiments; $*P<0.002$ of lane 2 respect to lane 1 and of lane 4 with respect to lane 3, as determined by Student's two-tailed *t*-test. The ratio in lane 1 was arbitrarily taken as one. (**B**) Immunofluorescence for D182A, c-Fos and the ER marker GRP78 in cells co-transfected to express HA-c-Fos and D182A-green fluorescent protein (GFP), quiescent (–FBS, left column) or induced to grow with FBS during 15 min (+FBS, right column), visualizing GFP, or HA with anti HA antibody, and the ER with anti GRP78 antibody. The magnified images in (c and d) insets are the merged images of HA-c-Fos and D182A-GFP from the boxed areas in (a and c), and (b and d), respectively; the magnified images in (a and b) insets are the boxed area of a and b, respectively. The third row shows ER labelling; inset in e and f are the magnified images of the boxed areas in (a and e), and (b and f), respectively, to show c-Fos/ER colocalization. The fourth row (merge) shows a merged image of the three labels to evidence colocalization sites. Note that c-Fos expression in transfected cells is constitutive.

On the other hand, reversible phosphorylation/dephosphorylation of proteins has emerged as a fundamental molecular switch that is much less time consuming than protein synthesis for rapid cell activation (Hunter, 2009). In the case of c-Fos, a body of information showing that both levels of control are operating during mitogen-dependent cell activation is emerging.

Herein, we have identified the first kinase and the first phosphatase capable of phosphorylating and dephosphorylating tyr residues of c-Fos. Information has also been obtained that indicates that the regulation of this reversible post-translational modification of c-Fos is imposed on the de-phosphorylation step rather than on the phosphorylation one. It has been shown that induction of cells to re-enter growth promotes the translocation of TC45 from the nucleus to the cytoplasm (Figure 8) and the concomitant activation of this phosphatase (Figure 9). Regarding c-Src regulation, in spite that many details of the activation of this kinase have emerged during the past years (reviewed in Hunter, 2009), basal activity seems sufficient to maintain the basal levels of c-Fos found in quiescent cells in its phosphorylated state (Figure 2).

In fact, that the regulation of the phenomenon is on the dephosphorylation step seems reasonable because dephosphorylation rather than phosphorylation will enable the cell to activate the signalling pathway leading to an increase in membrane biogenesis in response to extracellular mitogenic stimuli: dephosphorylated rather than phosphorylated c-Fos activates phospholipid synthesis. Consequently, cells could maintain phosphorylation of c-Fos constitutively activated so that the cell is metabolically arrested but ready to rapidly respond to growth stimuli. The more prolonged response of the cell would be achieved through the induction of the *de novo* synthesis of c-Fos, the other proposed mechanism to control c-Fos lipid-activating capacity. So, it can be envisaged a phosphorylation/dephosphorylation cycle of c-Fos as the rapid cell response to mitogen stimulation followed by the activation of the *de novo* synthesis of c-Fos to maintain the cells in a metabolically activated state. It still remains to be determined if the newly synthesized c-Fos also enters the phosphorylation/dephosphorylation cycle. It is hypothesized that the

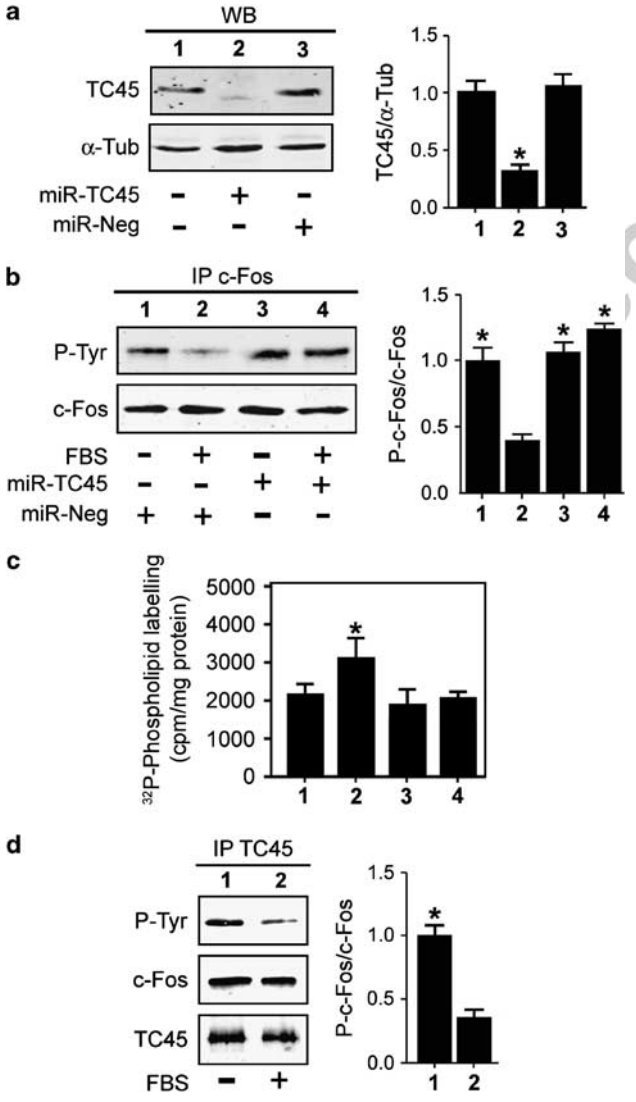

Figure 9 TC45 dephosphorylates c-Fos on addition of FBS to cell cultures. (**a**) Cells grown for 48 h in the presence of TC45 microRNA show effective reduction of TC45 expression with respect to those grown in the absence or in the presence of miR-Neg, as determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by WB using an anti TC45 antibody (upper row). The lower row shows α-tubulin staining used as a loading control. To the right are the optical density ratios of TC45/α-tubulin expressed as the mean ± s.d. of three experiments; TC45 was significantly reduced in miR-TC45-treated cells as compared either with non-treated cells or cells treated with miR-Neg; $*P<0.002$ as determined by Student's two-tailed *t*-test. The ratio in lane 1 was arbitrarily taken as one. (**b**) Content of P-c-Fos in quiescent and FBS-stimulated (15 min) cells in which TC45 expression was knocked down with miR-TC45 for 48 h. Cell lysates were subjected to IP with anti c-Fos antibody followed by SDS–PAGE and WB using anti-P-Tyr antibody (upper row) and compared with sister cells grown in the presence of miR-Neg. A control of c-Fos loaded in each lane is shown in the lower row. To the right are the optical density ratios of P-c-Fos/c-Fos expressed as the mean ± s.d. of three experiments; $*P<0.002$ with respect to lane 2, as determined by Student's two-tailed *t*-test. The ratio in lane 1 was arbitrarily taken as one. (**c**) Cells treated for 48 h with miR-TC45 or miR-Neg as in (**b**) were fed cycloheximide (50 μg/ml culture medium) for 30 min before changing medium to fresh medium containing or not FBS for 15 min as indicated; then cells were harvested and assayed for *in vitro* phospholipid synthesis capacity. Results are the mean c.p.m./mg protein of ^{32}P incorporated into phospholipids ± s.d. of three experiments performed in triplicate; $*P<0.002$ with respect to lane 1, as determined by Student's two-tailed *t*-test. (**d**) Cell lysates prepared from quiescent (−FBS) and growing cells (+ FBS for 15 min) were subjected to IP for TC45 and phosphatase activity determined using purified P-c-Fos. Incubates were subjected to SDS–PAGE and WB using anti-P-tyr (upper row) and anti c-Fos (lower row) antibodies. To the right are the optical density ratios of P-c-Fos/c-Fos. Results are the mean ratios ± s.d. of three experiments; $*P<0.001$ as determined by Student's two-tailed *t*-test. Note the higher phosphatase activity of TC45 obtained from growing cells as compared with that in quiescent cells. A control of IP TC45 added to each incubate is shown in the last row of Figure 9d.

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entry or not of c-Fos into the cycle when cells are metabolically activated is stochastic whereas tyr-phosphorylation of c-Fos could increase the half-life of this protein and in this way determine its fate either to degradation when not phosphorylated or to form the small pool of phosphorylated c-Fos contained in quiescent cells. Future studies are needed to clarify this aspect of the phenomenon.

Mitogen stimulation results in the post-translational activation of TC45 PTP. However, little is known about the molecular mechanism involved in this activation. It is clear, however, that part of the activation mechanism includes its translocation from its nuclear localization to the cytoplasm. In this case, translocation would enable its interaction with c-Fos to catalytically de-phosphorylate it. One regulatory mechanism reported for TC45 is on its positively charged C-terminus that negatively regulates its activity; a truncation mutant lacking this region is constitutively active (Hao *et al*., 1997). TC45 is also activated by the collagen-binding integrin $\alpha 1\beta 1$, which selectively interacts with the N-terminal of TC45, activating it in response to collagen adhesion (Mattila *et al*., 2005) whereas reversible oxidation inactivates TC45 (Meng *et al*., 2004). It will be of key interest to determine precisely the activation mechanism for TC45 because in this case, PTP acts as a proto-oncogene rather than a tumor-suppressor gene as has been reported for diverse PTPs (reviewed in Julien *et al*., 2011).

Together, these results support the notion that mitogen stimulation commands the activation of previously formed but inactive c-Fos because of its tyr-phosphorylated state thus promoting a rapid metabolic response in the cell. This first wave of response is followed by a second, more prolonged wave of metabolic stimulation achieved by the induction of the *de novo* synthesis of c-Fos (Gil *et al*., 2004; Portal *et al*., 2007). Both responses would converge to assure the activation of the cell metabolic machinery to enable high rates of membrane biogenesis.

Materials and methods

Cell culture conditions, transfections and extract preparation
NIH3T3 wt, knock-out for c-Src or for SYF and T98G cells (ATCC, Bethesda, MD, USA) were grown under standard culture conditions in Dulbecco's modified Eagle medium (Gibco, BRL) supplemented with 10% FBS. After desired confluence, growth was continued for 48 h in the absence of FBS to achieve quiescence. Cells were induced to re-enter growth by addition of 10% FBS (+FBS) for the time indicated in each case or continued to be cultured –FBS until harvesting.

Cell transfections were performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. For harvesting, cells were rinsed twice with ice-cold 10 mM phosphate-buffered saline (PBS) and harvested in modified RIPA buffer (50 mM Tris HCl pH 7.4, 50 mM NaF, 150 mM NaCl, 1% Nonidet P-40) for WB and IP analysis or in PBS for phospholipid analysis. Cell proliferatión was determined with CyQUANT NF Cell Proliferation Assay Kit (Invitrogen).

Electrophoresis, WB and IP assays
Cell lysates (10 μg) or IP's were fractionated through sodium dodecyl sulfate-containing polyacrylamide gels (12%) and electrotransferred at 300 mA during 1 h. Immuno-detection of c-Fos, Y10/30F, Y10/30E, TC-PTP, c-Src, P-tyr, HA and *c-myc* flag were carried at room temperature by blocking of membranes with 10 mM TBS containing 5% non-fat dried milk for 1 h, followed by incubation with c-Fos antibody (1/5000, Sigma-Aldrich, USA), TC-PTP antibody (1/500, Santa Cruz Biosciences, UK), c-Src antibody (1/500, eBioscience, USA), PY20 mAb (1/1000, Sigma-Aldrich), HA antibody (1/2000, Sigma-Aldrich) or c-myc antibody (1/1000, Sigma-Aldrich) overnight at 4 °C in TBS, 0.1% Tween 20. Membranes were washed three times (10 min) with TBS-Tween 20 and incubated 2 h at room temperature with IRDye 800CW goat anti-mouse secondary antibody or IRDye 800CW goat anti-rabbit secondary antibody (1/25000, LI-COR Bioscience). Membranes were washed and immuno-detection performed using ODYSSEY Infrared Imaging System (LI-COR Bioscience). For IP assays, 500 μg of total protein was subjected to IP with the desired antibody during 3 h at 4 °C, washed, cleared by centrifugation and the procedure continued as indicated in each case. When required, precipitates were recovered in Laemmli loading buffer.

In culture *phospholipid labelling*
To determine ^{32}P-phospholipid labelling *in culture*, T98G cells, transiently transfected to express c-Fos or its mutants Y10/30E or Y10/30F or not transfected, were grown an additional 48 h in the absence of FBS to attain quiescence. c-Fos ASO or SO oligonulceotides were added to the medium 3 h before FBS addition, as indicated (Gil *et al*., 2004). Cells were pulsed with 50 μCi/ml ^{32}P-orthophosphate (PerkinElmer Life Sciences, Boston, MA, USA) the last 2 h before harvesting and ^{32}P-phospholipid labelling determined (Guido and Caputto, 1990).

In vitro *phospholipid labelling*
In vitro phospholipid labelling by T98G cell homogenates was as described previously (Gil *et al*., 2004). Briefly, reactions were initiated by addition of 100 μg of cell homogenate and stopped by the addition of trichloroacetic acid and phosphotungstic acid (5–0.5% w/v, respectively).

Purification of recombinant proteins
His-tagged c-Fos (pDS56-HisFos), Y10/30F, Y10/30E (pET15b) were expressed as described previously (Borioli *et al*., 2001). Expression plasmids for c-Src and TC45, were kindly provided by MA Seeliger and H Charbonneau, respectively. Protein determination was according to Bradford, 1976.

Phosphorylation and dephosphorylation assays
To phosphorylate c-Fos, 2 μg of recombinant c-Fos were incubated with 5 ng/μl of recombinant c-Src in 30 μl of kinase buffer (20 mM Hepes pH 7.4, 1 mM $MgCl_2$, 1 mM DTT and 30 μM ATP). Incubations were for 30 min at 30°C and reactions stopped by the addition of 3X SB. To determine endogenous c-Src activity, 2 ug of c-Fos or enolase were incubated with c-Src IP as indicated above; phosphorylation was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and WB as described.

To determine phosphatase activity, membranes containing P-c-Fos were incubated in 1 ml phosphatase buffer (25 mM imidazol, 0.1% β-mercapto-ethanol) plus 2 ng/μl of recombi-

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nant TC45 for 30 min at 30°C; membranes were washed five times with TBS and bands visualized with PY20 antibody as described above. To determine endogenous TC45 activity, 2 μg of P-c-Fos were incubated with TC45 IP as indicated above.

Immunofluorescence microscopy

Cells grown on poly-Lysine (1 g/ml)-coated coverslips were rinsed twice with ice-cold 10 mM PBS and fixed in 3% (w/v) paraformaldehyde, 4% (w/v) sucrose in PBS 10 mM at 37 °C for 10 min. Washed cells were permeabilized with 0.25% Triton x-100 in PBS for 10 min at 37 °C, washed and blocked with blocking buffer (1% bovine serum albumin in 10 mM PBS) for 2 h and incubated over-night at 4 °C in blocking buffer containing c-Fos antibody (1/500), c-Src antibody (1/300), HA-antibody (1/500) and/or GRP78 antibody (1/300, Santa Cruz Biosciences) as indicated. Cells were then washed twice in 10 mM PBS, 0.1% Tween 20 and incubated with Alexa 546 anti-mouse antibody, Alexa 633 anti-rabbit antibody and/or Alexa 488 anti-rabbit antibody (1/1000, Molecular Probes, Eugene, OR, USA). Coverslips were Q11 mounted with FluorSave (Calbiochem, USA) and visualized with a 100 × objective using a Olympus FV1000 or Pascal 5 laser scanning confocal microscope using Olympus or Carl Q12 Zeiss software, respectively, for image analysis.

Reverse transcriptase–PCR, constructs and site directed mutagenesis

T98G total RNA was isolated following Trizol protocol (Invitrogen); 2 μg was used for reverse transcription with oligo dT primers according to M-MLV Reverse Transcriptase Q13 (Promega, USA) using the following primers:

TC45 or TC48 Fow 5′-CGGGATCCATGCCCACCACCA TCGA-3′;

TC45 Rev 5′-GGAATTCCAGGTGTCTGTCAATCTTGG CCT-3′;

TC48 Rev 5′-GGAATTCCATAGGGCATTTTGCTGAA AAAACAGTC-3′.

Plasmids containing TC45 *wt* or TC45-D182A kindly provided by T Tiganis were cloned in pCDNA3.1b (Invitrogen) in *Bam*HI y *Eco*RI site, and in pEGFP-N3 (Clontech, USA) in *Bgl*II y *Eco*RI site, using the primers described above. Q14

The BLOCK-iT Pol II miR RNAi Expression Vector Kit (Invitrogen) was used to clone miRNA sequence contained within the engineered pre-miRNA into pcDNA 6.2-GW/EmGFP-miR expression vector to generate expression clone miR-TC45. The miRNA oligo used as top strand was 5′-TGCTGTTTAGAGGAAAGTCCTGTACAGTTTTGGCCA CTGACTGACTGTACAGGTTTCCTCTAAA-3′.

c-Fos tyr residues #10 and #30 were mutated over pDS56His-Fos by the circular mutagenesis method (Sambrook and Russel, 2001).

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank HJF Maccioni for helpful discussions and H Carbonneau (Pardue University, West Lafayette, IN, USA) for providing pET-TC45a. pCG-TC45 and pCG-D182A were kind gifts from T Tiganis (Cold Spring Harbor Laboratory, New York, NY, USA) and pET-c-Src from MA Seelinger (University of California, Berkeley, CA, USA). Excellent technical assistance from Susana Deza and Gabriela Schachner is acknowledged. This work was supported by grants from the ANPCyT and CONICET, Ministerio de Ciencia, Tecnología e Innovación Productiva de Argentina, SECyT-Universidad Nacional de Córdoba, Argentina and the JS McDonnell Foundation. BLC is a career member and GOF and FV are fellows of CONICET.

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Uncorrected Proof