Tumour necrosis factor- α activation of protein kinase B in WEHI-164 cells is accompanied by increased phosphorylation of Ser⁴⁷³, but not Thr³⁰⁸

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Tumour necrosis factor- α (TNF- α) may activate both cell survival and cell death pathways. In the murine fibrosarcoma cell line WEHI-164, physiological concentrations (1 ng/ml) of TNF- α induced wortmannin-sensitive cell ruffling characteristic of the phosphoinositide 3-kinase (PI3-kinase) activation associated with cell survival. Wortmannin also enhanced cell death induced by TNF- α in the presence of actinomycin D, confirming that TNF- α activates a transcription-independent survival pathway requiring PI3-kinase activity. Both TNF- α and insulin-like growth factor 1 (IGF-1) caused a 6–10-fold wortmannin-sensitive increase in protein kinase B (PKB) activity within 5 min. For IGF-1, this was associated with an increase in phosphorylation of both Thr³⁰⁸ and Ser⁴⁷³, whereas for TNF- α only phosphorylation of Ser⁴⁷³ was increased, even in the presence of okadaic acid to inhibit protein phosphatases 1 and 2A. TNF- α did not decrease the phosphorylation of Thr³⁰⁸ induced by IGF-1, implying that TNF- α neither inhibits phosphoinositide-dependent kinase 1 (PDK1) nor activates an opposing phosphatase. In WEHI cells

overexpressing a form of PKB, IGF-1 increased phosphorylation of Ser⁴⁷³ on PKB, but not its kinase activity, whereas TNF- α failed to induce Ser⁴⁷³ phosphorylation or kinase activation of either overexpressed T308A or wild-type PKB (where T308A is the mutant bearing the substitution Thr³⁰⁸ $\rightarrow$ A). IGF-1 caused translocation of green-fluorescent-protein-tagged ADP-ribosylation factor nucleotide-binding site opener (ARNO) to the plasma membrane of WEHI cells, but this was not detected with TNF- α . We conclude that, at physiological concentrations, TNF- α activates endogenous PKB by stimulating PDK2 (increase in Ser⁴⁷³ phosphorylation) in a PI3-kinase-dependent (wortmannin-sensitive) manner, without causing detectable stimulation of PDK1 (no increase in Thr³⁰⁸ phosphorylation) or ARNO translocation. Possible explanations of these observations are discussed.

Key words: ADP-ribosylation factor nucleotide-binding site opener, cell survival, insulin-like growth factor 1, phosphoinositide-dependent kinase, phosphoinositide 3-kinase.

INTRODUCTION

Protein kinase B (PKB) is the cellular homologue of the viral oncogene product v-Akt, encoded in the genome of the retrovirus AKT-8, and was originally isolated from a rodent T cell lymphoma [1–3]. It is a 57 kDa serine/threonine protein kinase that has been shown to play key roles in insulin signalling, control of the cell cycle and inhibition of apoptosis [4,5]. Activation of PKB by insulin is mediated via phosphorylation of Thr³⁰⁸ in the activation loop of the kinase domain, and Ser⁴⁷³ in the C-terminal regulatory region; both events are dependent on phosphoinositide 3-kinase (PI3-kinase) activity. The relationship between phosphorylation status and activation of PKB by insulin and insulin-like growth factor 1 (IGF-1) has been studied by substitution of the serine and threonine residues with alanine or aspartate respectively. These studies indicate that single phosphorylation of either site alone leads to partial activation *in vitro*, whereas dual phosphorylation of both sites is synergistic in nature, and is required for maximal activation. In addition, each phosphorylation event is independent of the other *in vivo* [6]. In contrast, studies using platelet-derived growth factor ('PDGF') as the agonist have suggested that Thr³⁰⁸ phosphorylation is an absolute requirement for PKB activation *in vivo*. Thus phosphorylation of Ser⁴⁷³ alone was found to be unable to generate activity, but was necessary for maximal activation [7].

The signal-transduction pathway that leads to activation of PKB involves activation of PI3-kinase and formation of PtdIns(3,4,5)P₃ at the plasma membrane. PKB translocates from the cytosol to interact with this lipid via its pleckstrin homology domain, thereby allowing a conformational change that exposes its phosphorylation sites [8,9]. Thr³⁰⁸ is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1), a 63 kDa serine/threonine kinase that is expressed ubiquitously, which appears to be constitutively active under basal conditions [10,11]. The kinase that phosphorylates Ser⁴⁷³ (termed PDK2) has yet to be identified, although it has been suggested that PDK1 can acquire PDK2 activity via protein–protein interactions [12]. Other candidate proteins suggested are p38 kinase-dependent mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK-2) [13] and integrin-linked kinase [14].

It was recently shown that tumour necrosis factor- α (TNF- α) causes phosphorylation and activation of PKB in a PI3-kinase-dependent manner [15,16]. TNF- α is a pro-inflammatory cytokine, produced primarily by activated macrophages, which utilizes two ubiquitously expressed cell-surface receptors, p55/TNFR-B and p75/TNFR-A, to engage an array of interlinked signalling pathways [17]. Its effects depend on cell type and conditions, but range from survival (proliferation and differentiation) to cytotoxicity (apoptosis and necrosis) [18,19]. Survival

Abbreviations used: ARNO, ADP-ribosylation factor nucleotide-binding site opener; CHO, Chinese hamster ovary; ECL, enhanced chemiluminescence; GFP, green fluorescent protein; GSK-3, glycogen synthase kinase 3; IGF-1, insulin-like growth factor 1; MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; NF- κ B, nuclear factor- κ B; PDK1/2, phosphoinositide-dependent kinase 1/2; PI3-kinase, phosphoinositide 3-kinase; PKB, protein kinase B; T308A, mutant bearing the substitution Thr³⁰⁸ $\rightarrow$ Ala, etc.; TNF- α , tumour necrosis factor- α .

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pathways are thought to be mediated primarily by the transcription factor nuclear factor- κ B (NF- κ B), which controls the expression of many genes involved in inflammation and immune responses. NF- κ B has been shown to be a target for PKB in fibroblast-derived cell lines [20]. In contrast, human endothelial cells appear to possess a TNF- α -induced, PKB-dependent survival pathway that does not require NF- κ B activation [21]. Thus, although PKB clearly represents a survival or anti-apoptotic signal, its precise role in the context of TNF- α signalling remains to be elucidated.

In the present paper, we investigate the phosphorylation and activation of both endogenous and overexpressed PKB in the murine fibrosarcoma cell line WEHI-164 stimulated with physiological concentrations (1 ng/ml) of TNF- α , as opposed to the supramaximal levels used by many workers. At these low concentrations of TNF- α , WEHI-164 cells undergo apoptosis, provided that the NF- κ B-dependent survival pathways are inhibited by addition of the transcription inhibitor actinomycin D. We confirm that TNF- α activates PKB in these cells via a PI3-kinase-dependent pathway. However, in contrast with the effects of IGF-1, this activation is accompanied by phosphorylation of Ser⁴⁷³ without any detectable increase in Thr³⁰⁸ phosphorylation. Our data suggest that, at physiological concentrations, TNF- α activates endogenous PKB by stimulating PDK2 (increase in Ser⁴⁷³ phosphorylation), but not PDK1 (no increase in Thr³⁰⁸ phosphorylation). However, the wortmannin sensitivity of this response implies that PDK2 activation still requires some activity of PI3-kinase. These conclusions are entirely consistent with recent data from experiments with Chinese hamster ovary (CHO) cells overexpressing PDK1 [22]. In these cells, insulin promoted a rapid phosphorylation of Thr³⁰⁸ on PKB that was totally lost after 5 min, although Ser⁴⁷³ phosphorylation and PKB kinase activity remained elevated, implying that transient phosphorylation of Thr³⁰⁸ is sufficient for PKB activation.

EXPERIMENTAL

Materials

Human recombinant TNF- α was obtained from Amersham Pharmacia, and human recombinant IGF-1 was from Calbiochem. WEHI-164 cells were purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). Tissue-culture reagents and media were supplied by Gibco. For detection of total PKB by Western blotting, anti-PKB α serum raised in the rabbit against a synthetic peptide corresponding to residues 465–480 of human PKB α , provided by Dr Emily Foulstone (Department of Biochemistry, University of Bristol, U.K.), was employed, as described previously [23]. At the dilution used (1:1000), this detected only a single broad band of 60 kDa, which corresponded to PKB in its different phosphorylation states. Detection of PKB phosphorylated on Thr³⁰⁸ and Ser⁴⁷³ utilized affinity-purified polyclonal antibodies that were raised against the appropriate phosphopeptides. These antibodies were obtained from New England Biolabs; further details of their characterization may be found at <http://www.neb.com>. For immunoprecipitation of PKB from cell extracts, rabbit polyclonal antisera against synthetic peptides corresponding to residues 453–470 of murine PKB α and PKB β were raised. These antibodies detected the same 60 kDa band on Western blotting as the anti-PKB antibodies described above, and binding was prevented by peptide competition. These antibodies also quantitatively immunoprecipitated PKB activity measured against defined PKB substrates. Immunoprecipitating antibodies against EE- and Myc-tagged proteins (where 'EE' represents an acidic cluster of sequence MEEEFMPMEF, and the Myc epitope is

MEQKLISEEDLEF) were generously given by Dr Harry Mellor (Department of Biochemistry, University of Bristol, U.K.). Bovine PKB α cDNA ligated into the transient expression vector pCMV with either N-terminal EE or Myc tags [7] was kindly donated by Dr Phillip Hawkins (Babraham Institute, Cambridge, U.K.). All other chemicals and reagents were obtained from Sigma.

Methods

Cell culture

WEHI-164 cells were cultured in RPMI 1640 medium [supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (0.1 mg/ml) and glutamine (0.3 mg/ml)] in a humidified atmosphere of air/CO₂ (19:1) at 37 °C. Cells were maintained in exponential phase growth and were used below passage 40.

Confocal microscopy

WEHI-164 cells were cultured overnight on 13 mm glass coverslips coated with human fibronectin (5 μ g/cm²) in 24-well plates at an initial density of 1×10^5 cells/well. After appropriate treatments, they were fixed in 3% (w/v) formaldehyde for 25 min at 4 °C and then permeabilized in 0.2% (w/v) Triton X-100 for 5 min at room temperature. Cells were stained with tetramethylrhodamine β -isothiocyanate ('TRITC')-phalloidin (diluted 1:1000) for 30 min, before washing in PBS, mounting in Moviol and viewing with a Leica TCS-NT confocal laser scanning microscope.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) cell survival assay

WEHI-164 cells were grown in 96-well culture dishes at a density of 5×10^4 cells/well. After incubation with the desired effectors, cells were washed once in PBS and incubated with MTT (0.5 mg/ml in PBS; 200 μ l/well) for 3 h in a humidified incubator at 37 °C with 5% CO₂. MTT solution was then removed by gently tapping the plate on to an absorbent surface, and 100 μ l of DMSO was added to each well to dissolve the crystalline product for measurement of A_{540} .

Detection of PKB phosphorylation by Western blotting

WEHI-164 cells were cultured in 25 cm² flasks to approx. 70–80% confluence, before initiating the desired treatment. After washing once in PBS, cells were harvested by scraping and centrifugation at 1000 *g* for 3 min. Pellets were resuspended in 100 μ l of ice-cold PKB lysis buffer [120 mM NaCl/50 mM Hepes (pH 7.4)/10 mM NaF/1 mM EDTA/40 mM β -glycerophosphate/1% (v/v) Nonidet P40/1 μ M microcystin LR/0.1 mM PMSF/1 mM sodium vanadate/10 μ M leupeptin/1 μ M pepstatin/10 μ M antipain], and the samples were centrifuged at 13000 *g* for 3 min at 4 °C to remove insoluble material. Supernatants were frozen in liquid nitrogen and stored at –80 °C until use. Samples were separated on SDS/8% (w/v) polyacrylamide electrophoresis gels, and proteins were electroblotted on to Immobilon membranes (Millipore). Membranes were blocked for 1 h at room temperature in blocking buffer [PBS containing 0.1% (v/v) Tween 20/5% (w/v) skimmed-milk powder], before incubation with primary antibodies diluted 1:1000 [incubation time of 1 h for Ser⁴⁷³ $\rightarrow$ Pro (S473P) and total PKB; overnight for Thr³⁰⁸ $\rightarrow$ Pro (T308P)]. Horseradish-

peroxidase-conjugated secondary antibody was used at a dilution of 1:10000 for 1 h, followed by detection by an enhanced chemiluminescence (ECL) method. Quantification of blots was achieved by densitometry. For ECL, several exposures of the film were employed to ensure that quantification by scanning was in the linear region.

Assay of PKB activation by phosphorylation of glycogen synthase kinase 3 (GSK-3) fusion protein

This was performed using the Akt kinase assay kit (New England Biolabs) according to the manufacturer's instructions, and further details of the technique and reagents used are available at <http://www.neb.com>. Briefly, cytosolic extracts were prepared as described above, and PKB was immunoprecipitated by incubation with PKB mouse monoclonal antibody (20 μ l per 200 μ l sample) immobilized on agarose hydrazide beads for 3 h at 4 °C. Beads were harvested by centrifugation and washed twice in PKB lysis buffer, before resuspending in kinase buffer containing 200 μ M ATP and 1 μ g of GSK-3 fusion protein. The reaction was terminated by addition of SDS sample buffer, beads were pelleted by microcentrifugation, and the supernatants were boiled for 5 min before SDS/PAGE and Western blotting with phospho-(GSK-3 α/β) antibody [1:1000 dilution overnight at 4 °C in PBS containing 0.2% (v/v) Tween 20 and 5% (w/v) BSA] were performed. Horseradish-peroxidase-conjugated secondary antibody was used at a dilution of 1:2000 for 1 h, followed by detection by ECL.

Assay of PKB activation using a synthetic peptide substrate

PKB activity was determined more quantitatively by measuring the incorporation of [32 P] P_i into a synthetic peptide substrate designed on the basis of the consensus sequence for the kinase, RPRATF, and synthesized by Graham Bloomberg (University of Bristol, U.K.). Briefly, cell extracts were prepared from the same quantity of cells for each treatment as described above, and the α and β isoforms of PKB were immunoprecipitated using a 1:500 dilution of anti-PKB α and anti-PKB β sera in the presence of Protein A–Sephadex. Immunoprecipitates were washed twice and then resuspended in assay buffer [20 mM Hepes (pH 7.5)/20 mM β -glycerophosphate/1 mM EDTA/0.1% (v/v) 2-mercaptoethanol/200 μ M cAMP-dependent protein kinase inhibitor peptide, IP20]. The activity of PKB in the immunoprecipitates was determined in the presence of [γ - 32 P]ATP (100 μ M; 200–500 c.p.m./pmol), MgCl₂ (2 mM) and substrate peptide (0.5 mg/ml) for 15 min at 30 °C.

Transient transfection

WEHI cells were cultured in 25 cm² flasks until they were 60–70% confluent. TFX-50 reagent (Promega) was used at a ratio of 4.5 μ l/ μ g DNA. Each cell sample was transfected with 1 μ g of DNA for 3 h (without serum starvation), after which the medium was replaced and cells were cultured overnight before processing.

Visualization of ADP-ribosylation factor nucleotide-binding site opener (ARNO) translocation using time-lapse confocal microscopy

The generation of 3-phosphorylated lipids in the plasma membrane of WEHI-164 cells in response to IGF-1 or TNF- α was visualized using a green fluorescent protein (GFP)-tagged catalytically dead mutant of the high-affinity PtdIns(3,4,5) P_3 -binding protein, ARNO. Cells were transiently transfected as above and

starved for 6 h before microscopy. Live imaging was performed at 37 °C in KH buffer [5 mM Hepes/10 mM glucose/25 mM NaHCO₃/1.2 mM K₂HPO₄/118 mM NaCl/4.7 mM KCl/1.2 mM MgSO₄/1.3 mM CaCl₂ (pH 7.4)] using a Wallac Ultra-View confocal microscope (PerkinElmer Life Sciences), as described previously [24].

RESULTS

TNF- α activates a PI3-kinase-mediated survival pathway in WEHI-164 cells

In most cases studied, growth factor stimulation of PKB is dependent on type I PI3-kinase activity [11]. The data shown in Figure 1(a)–1(d) show that TNF- α induces typical growth-factor-mediated morphological changes in WEHI-164 cells. Thus membrane ruffling and lamellipodia formation (effects known to be associated with PI3-kinase activity) began to be seen within 10 min of treatment, and reached a maximum at approx. 30 min. These changes occurred both in the presence and absence of the transcription inhibitor actinomycin D (results not shown), confirming that the TNF- α -induced 'survival pathways' downstream of NF- κ B activation are not required for this rapid response. However, pretreatment with wortmannin, an irreversible inhibitor of type I PI3-kinase, completely abolished the membrane ruffling response, although it did cause some morphological changes in its own right (Figure 1b).

Figure 1 TNF- α induces PI3-kinase-dependent membrane ruffling in WEHI cells

Cells were cultured, fixed and stained with tetramethylrhodamine β -isothiocyanate ('TRITC')-phalloidin as described in the Experimental section. Normal morphology in the untreated cells is shown in (a), whereas in (c) cells were treated with TNF- α (1 ng/ml) plus actinomycin D (0.5 μ g/ml) for 30 min before fixation. Membrane ruffling with lamellipodia formation is indicated by the arrows in (c). (b) and (d) Cells were treated with wortmannin (100 nM) either alone (b) or 15 min before TNF- α /actinomycin D treatment for 30 min (d). Scale bars represent 10 μ m.

Figure 2 Effects of wortmannin and actinomycin D (ActD) on cell survival in the presence and absence of TNF- α

WEHI cells were cultured in 96-well plates and pretreated with wortmannin (100 nM), actinomycin D (0.5 μ g/ml) or both for 15 min before addition of 1 ng/ml TNF- α . After 16–20 h, cells were washed once in PBS and cell survival was estimated by the MTT assay as described in the Experimental section. An arbitrary value of 100% was assigned to control (untreated) cells. Error bars signify the S.E.M. for eight replicates, and data shown are representative of three experiments, each yielding similar results.

In order to ascertain whether this PI3-kinase-dependent pathway operates to enhance cell survival via a transcription-dependent or -independent mechanism, we used the MTT assay [25] to estimate the survival of cell populations exposed to TNF- α following pretreatment with wortmannin, actinomycin D or both (Figure 2). Wortmannin alone enhanced TNF- α toxicity by approx. 20% in the absence of actinomycin, but had a greater effect (55%) in the presence of actinomycin. Similar results were seen with the alternative PI3-kinase inhibitor LY294002 (results not shown). These results imply that in WEHI cells there are at least two survival pathways activated by TNF- α : a transcription-dependent mechanism, probably involving NF- κ B, that is largely independent of PI3-kinase activation, and a transcription-independent mechanism that is downstream of PI3-kinase activation.

TNF- α phosphorylates endogenous PKB at Ser⁴⁷³ in a PI3-kinase-dependent manner without detectable phosphorylation of Thr³⁰⁸

To investigate TNF- α -mediated events downstream of PI3-kinase activation, we used phospho-specific antibodies with Western blotting to detect the phosphorylation state of endogenous PKB (Figures 3 and 4). The phosphorylation of Ser⁴⁷³ was barely detectable in the basal state, but a large increase in phosphorylation was observed within 5 min of exposure to TNF- α , and this was sustained for at least 2 h, reaching a maximum at around 30 min (Figures 3a and 4). This effect was demonstrable without the need for serum starvation. In contrast, no detectable phosphorylation of Thr³⁰⁸ was induced by TNF- α (Figures 3d and 4), although phosphorylation of this site was readily detected in response to IGF-1, which was employed at a supra-physiological concentration (100 ng/ml) as a positive control (Figures 3b, 3d and 4). Pretreatment of cells with the PI3-kinase inhibitor wortmannin abolished completely both basal Ser⁴⁷³ phosphorylation and that induced by TNF- α (Figure 3c). Wortmannin also inhibited the phosphorylation of both Ser⁴⁷³ and Thr³⁰⁸ induced by IGF-1 (results not shown). Similar results were seen with the alternative PI3-kinase inhibitor, LY294002 (results also not shown).

Figure 3 TNF- α phosphorylates PKB at Ser⁴⁷³ in a PI3-kinase-dependent manner without detectable phosphorylation of Thr³⁰⁸

Cells were incubated for the times shown with TNF- α (1 ng/ml + actinomycin D, 0.5 μ g/ml) or IGF-1 (100 ng/ml), as indicated. Samples for Western blotting were prepared as described in the Experimental section, and membranes were probed sequentially with specific antibodies against the Thr³⁰⁸- and Ser⁴⁷³-phosphorylated forms of PKB and total PKB (S473-P and T308-P respectively). In the experiment shown in (a), no phosphorylation of Thr³⁰⁸ was observed in response to TNF- α , and thus the results are not shown. In panel (c), wortmannin was added at 100 nM where indicated. Cont, control.

Activation of endogenous PKB by TNF- α is comparable with that mediated by IGF-1

We used two methods to demonstrate that TNF- α increases the activity of endogenous PKB despite the absence of detectable Thr³⁰⁸ phosphorylation. First, we measured phosphorylation of a known downstream target of PKB; namely, GSK-3. Figure 5(a) indicates that PKB extracted from cells treated with TNF- α for 10 and 30 min caused phosphorylation of a GSK-3 fusion protein. Phosphorylation was readily detectable without serum starvation, but was prevented if wortmannin was added with the TNF- α . Activation of PKB was quantified further by measuring the phosphorylation of an appropriate peptide substrate with [γ -³²P]ATP using the method of Cross et al. [4]. IGF-1 (100 ng/

Figure 4 Quantification of the phosphorylation of PKB at Ser⁴⁷³ and Thr³⁰⁸ induced by IGF-1 and TNF- α

Data from several experiments similar to those shown in Figure 3 were analysed by densitometry of the ECL blots. Band intensity was normalized by reference to the total PKB signal. Error bars represent the S.E.M. for the number of experiments shown in parentheses above the bars.

Figure 5 Activation of endogenous PKB by TNF- α

(a) Cytosolic extracts were prepared from cells incubated in the presence or absence of TNF- α (1 ng/ml + actinomycin D at a concentration of 0.5 μ g/ml) for 10 and 30 min, and wortmannin (100 nM), as indicated. PKB activation was determined by measuring phosphorylation of a GSK-3 fusion protein as described in the Experimental section. More quantitative data for PKB activation are shown in (b), determined using incorporation of [³²P]_i into a synthetic peptide substrate, as described in the Experimental section. Activation by TNF- α is compared with that induced by IGF-1 (100 ng/ml) at the same time. Data are expressed as the fold increase over control (no agonist) and as the means $\pm$ S.E.M. (error bars) of five separate experiments. Cont, control.

ml) caused a 7-fold increase in endogenous PKB activity within 5 min, reaching a maximum of 11-fold at 30 min (Figure 5b), at which point phosphorylation of Thr³⁰⁸ (but not of Ser⁴⁷³) had declined from its peak value at 5 min (Figure 3b). Surprisingly, activity induced by TNF- α closely matched these levels at each time point, despite the lack of Thr³⁰⁸ phosphorylation. These

Figure 6 TNF- α does not inhibit the phosphorylation of Thr³⁰⁸ induced by IGF-1

WEHI cells were treated with TNF- α (1 ng/ml + actinomycin D at a concentration of 0.5 μ g/ml), IGF-1 (100 ng/ml) or both for the times indicated. Cytosolic extracts were prepared and analysed by Western blotting as described in the legend to Figure 3. Cont, control.

Figure 7 Effects of okadaic acid on phosphorylation of Thr³⁰⁸ and Ser⁴⁷³

WEHI cells were pretreated with okadaic acid (1 μ M) for 30 min before the addition of TNF- α (1 ng/ml + actinomycin D at a concentration of 0.5 μ g/ml). Cytosolic extracts were prepared and analysed by Western blotting as described in the legend to Figure 3. Panels shown in (a) represent the same membrane stripped and re-probed with appropriate antibody, whereas quantification of the data provided from several experiments is shown in (b), performed as described in the legend to Figure 4.

data contrast with previous studies reporting that single-site phosphorylation of PKB *in vitro* only results in partial activation of the enzyme [6], but are consistent with recent data from experiments with CHO cells overexpressing PDK1 [22]. In these cells, insulin promoted a rapid phosphorylation of both Ser⁴⁷³ and Thr³⁰⁸, coincident with PKB activation, but Thr³⁰⁸ was subsequently dephosphorylated without loss of kinase activity or dephosphorylation of Ser⁴⁷³.

Figure 8 Effects of IGF and TNF- α on ARNO translocation to the plasma membrane

WEHI-164 cells were transiently transfected with the GFP-tagged catalytically 'dead' mutant of ARNO, and starved for 6 h before imaging using time-lapse confocal microscopy, as described in the Experimental section. IGF-1 (100 ng/ml) or TNF- α (1 ng/ml) (**a** and **b** respectively) were added 30 s after the initiation of imaging, and LY290042 (50 μ M) was added after a further 190 s. The frames shown were taken at 3 s, 135 s and 270 s, and demonstrate progressive bleaching of the GFP. A video showing the complete sequence of images for IGF-1 may be viewed at <http://www.BiochemJ.org/bj/359/bj3590119add.htm>. (**c**) Cells fixed after 30 min exposure to IGF-1 (100 ng/ml) or TNF- α (1 ng/ml) were then stained with tetramethylrhodamine β -isothiocyanate ('TRITC')-phalloidin to detect actin filaments before visualization of the GFP-ARNO and phalloidin by confocal microscopy.

TNF- α does not prevent sustained phosphorylation of Thr³⁰⁸ by activation of a protein phosphatase or secondary inhibition of PDK1

Since others have demonstrated an absolute dependence of PKB activation on Thr³⁰⁸ phosphorylation *in vivo* [7], it seemed possible that TNF- α might initially stimulate phosphorylation of both Thr³⁰⁸ and Ser⁴⁷³, but that the former occurs only transiently. It might then be followed by dephosphorylation, as found following insulin stimulation of CHO cells overexpressing PDK1 [22]. However, even after exposure to TNF- α for only 1 min, at which time phosphorylation induced by IGF-1 was clearly visible, we were unable to demonstrate phosphorylation of this site in response to TNF- α (results not shown). Nevertheless, this would not rule out the possibility that, at the same time as activating the kinase that phosphorylates Ser⁴⁷³ (PDK2), TNF- α might subsequently either inhibit the kinase that phosphorylates Thr³⁰⁸ (PDK1) or activate an opposing phosphatase. If either effect were substantial, TNF- α should decrease the phosphorylation of Thr³⁰⁸ induced by IGF-1 when both agents are added together. This was not the case, since 10 min after IGF-1 addition the presence of TNF- α had no effect on Thr³⁰⁸ phosphorylation, although after 30 min some decrease may occur (Figure 6). Our data could also be explained if there is a constitutively active protein phosphatase that is sufficiently active to dephosphorylate Thr³⁰⁸ as fast as PDK1 phosphorylates it when stimulated by TNF- α , but not when stimulated by IGF-1. Indeed, the transient

phosphorylation of Thr³⁰⁸ during insulin stimulation of CHO cells overexpressing PDK1 has been explained in this way [22]. Our data (Figure 3b) show a time-dependent decline in phosphorylation of Thr³⁰⁸ (but not that of Ser⁴⁷³), following an initial peak 5 min after IGF-1 addition, that is consistent with the activation of such a phosphatase. However, even in the presence of 1 μ M okadaic acid, which will serve to inhibit both protein phosphatases 1 and 2A at this concentration, no additional phosphorylation of Thr³⁰⁸ was induced by TNF- α , despite basal levels of Thr³⁰⁸ and Ser⁴⁷³ phosphorylation being enhanced (Figure 7). Thus, taken together, our data argue against any activation of PDK1 by TNF- α . This conclusion was strengthened by studies on the effects of TNF- α and IGF on the translocation of ARNO to the plasma membrane, a surrogate marker for PI3-kinase activation [24].

IGF-1, but not TNF- α , induces translocation of ARNO to the plasma membrane

In order to demonstrate any difference in the magnitude and time course of PI3-kinase activation between IGF-1 and TNF- α , we used GFP-ARNO, a known high-affinity PtdIns(3,4,5) P_3 -binding protein, as a specific reporter for 3-phosphorylated inositol lipid production. IGF-1 treatment caused a rapid recruitment of ARNO to the plasma membrane of WEHI-164 cells that was inhibited by the PI3-kinase inhibitor, LY290042 (Figure 8a). In contrast, there was no detectable translocation of GFP-ARNO

Figure 9 Effects of TNF- α and IGF-1 on phosphorylation and activity of the overexpressed wild-type (WT) and T308A mutant form of PKB

WEHI cells were transfected with either WT PKB or the site-directed mutant T308A, and treated with either IGF-1 (100 ng/ml) or TNF- α (1 ng/ml + actinomycin D at a concentration of 0.5 μ g/ml) for 30 min (b) or for the times indicated (a). Cytosolic extracts were prepared and analysed by Western blotting, as described in the legend to Figure 3 (a) or PKB-immunoprecipitated using anti-EE and anti-Myc antibodies, and activity was determined by incorporation of [32 P]P_i into a synthetic peptide substrate, as described in the legend to Figure 5. Data in (b) are shown as means $\pm$ S.E.M. (error bars) of five separate experiments.

in response to TNF- α up to 30 min after exposure (Figure 8b), despite the appearance of morphological changes between 10 and 30 min after TNF- α addition (Figure 8c). It should be noted that, for IGF-1 addition to the transfected cells, the translocation of GFP-ARNO is less pronounced after 30 min (Figure 8c) than after 2 min (Figure 8a), although ruffling was still apparent. These morphological changes, like those in untransfected cells (Figure 1), were sensitive to PI3-kinase inhibitors (results not shown).

PKB activation by TNF- α may require Thr³⁰⁸ phosphorylation

In order to investigate further whether or not phosphorylation of Thr³⁰⁸ is a prerequisite for PKB activation, despite not being enhanced by TNF- α , we determined the effects of TNF- α and IGF-1 on the phosphorylation and activation of overexpressed wild-type PKB and a T308A site-directed mutant (Figure 9). It was found that IGF-1 greatly stimulated phosphorylation of Ser⁴⁷³ in the absence of Thr³⁰⁸ (Figure 9a), in agreement with published work that each site can be phosphorylated independently *in vivo* [6]. Furthermore, activity measurements (Figure 9b) confirmed that activation of PKB does require Thr³⁰⁸ phosphorylation, because IGF-1 produced a 6-fold increase in activity of wild-type PKB, but no activation of the T308A mutant. However, in the case of TNF- α , we were unable to demonstrate either Ser⁴⁷³ or Thr³⁰⁸ phosphorylation, even in those cells overexpressing wild-type PKB (Figure 9a); nor could we detect any significant activation of PKB in either wild type or

T308A PKB-transfected cells (Figure 9b). It should be noted that the blots shown in Figure 9(a) were developed to reveal the phosphorylation of overexpressed PKB induced by IGF-1. Longer exposure revealed that phosphorylation of Ser⁴⁷³ occurred even in control cells, but still no increase was observed in response to TNF- α (results not shown). Thus it appears that the levels of basal phosphorylation of overexpressed PKB are sufficiently high, even with serum starvation, to mask any small increase induced by TNF- α . However, it should be noted that overexpression of both wild-type and T308A PKB caused profound morphological changes in WEHI cells (results not shown), and these might have resulted in an altered response to TNF- α .

DISCUSSION

We have demonstrated that TNF- α activates endogenous PKB in a fibroblast-derived cell line and, as with other agonists, this activation is wortmannin-sensitive and thus dependent on the action of PI3-kinase. However, unlike the situation with IGF-1, TNF- α activation is associated with an increase in phosphorylation of only Ser⁴⁷³, without a detectable increase in phosphorylation of Thr³⁰⁸. Even following stimulation of PKB activity with IGF-1, phosphorylation of Thr³⁰⁸ was not fully maintained after the initial peak at 5 min (Figure 3), yet kinase activity and Ser⁴⁷³ phosphorylation remained elevated (Figure 5). Thus it is clear that PKB activity is not dependent on a sustained increase in Thr³⁰⁸ phosphorylation. In the case of TNF- α , a transient stimulation of Thr³⁰⁸ seems unlikely, since even at the shortest times employed (1 min) no increase in Thr³⁰⁸ phosphorylation was observed. A coincident activation of a protein phosphatase might explain this, as has been proposed for the transient phosphorylation of Thr³⁰⁸ during insulin stimulation of CHO cells overexpressing PDK1 [22]. However, TNF- α did not cause an increase in Thr³⁰⁸ phosphorylation, even in the presence of the protein phosphatase inhibitor okadaic acid, which enhanced its basal phosphorylation (Figure 7). Furthermore, TNF- α treatment did not decrease the level of phosphorylation of Thr³⁰⁸ induced by IGF-1 (Figure 6) that would be predicted if it either activated a protein phosphatase or inhibited PDK1 following an initial activation. Reports that TNF- α can activate a protein phosphatase that dephosphorylates Ser⁴⁷³ via a ceramide-dependent mechanism [26] are also incompatible with our data.

The simplest conclusion from our data is that, at the physiological concentrations of TNF- α used here, PDK2 activity is stimulated independently of PDK1. However, this stimulation must involve some activation of PI3-kinase because limited membrane ruffling is observed (Figure 1), and both this and PKB activation are prevented by wortmannin. This might reflect a stimulation of PI3-kinase by TNF- α that is sufficient to activate both PDK2 and the mechanisms responsible for membrane ruffling, such as activation of Rac [27], but which is insufficient either to activate PDK1 (no Ser⁴⁷³ phosphorylation; Figure 3) or to induce detectable GFP-ARNO translocation (Figure 8). However, this explanation cannot account for why the overexpressed T308A mutant of PKB can be phosphorylated (but not activated) by IGF-1, but not by TNF- α . It is possible that there are two distinct PDK2 activities, one stimulated by IGF-1 that is active on the T308A mutant and another, inactive against this mutant, that is stimulated by TNF- α . Even in the former case, a conformational change caused by the T308A mutation must prevent PKB kinase being activated following Ser⁴⁷³ phosphorylation.

Another explanation of our data would be that TNF- α stimulates PDK2 to phosphorylate Ser⁴⁷³ on PKB only when Thr³⁰⁸ has already been phosphorylated by the basal activity of PDK1. This might reflect binding of PKB via its pleckstrin homology domain to small amounts of PtdIns(3,4,5)P₃ present in the plasma membrane even of unstimulated cells [8]. Such steady-state levels of Thr³⁰⁸ phosphorylation might be too small to detect, unless the protein phosphatase responsible for Thr³⁰⁸ dephosphorylation is blocked with okadaic acid. Once Ser⁴⁷³ has been phosphorylated, the resulting conformational change in PKB responsible for its activation could be maintained even following subsequent dephosphorylation of Thr³⁰⁸, provided that Ser⁴⁷³ remains phosphorylated. A similar conclusion has also been reached by others [22]. Our observation that TNF- α did not activate overexpressed PKB in WEHI-164 cells, despite activating endogenous PKB, is also compatible with this proposal. Thus, the small amounts of PtdIns(3,4,5)P₃ present in the plasma membrane of unstimulated cells might be saturated with endogenous PKB. If this were the case, with endogenous PKB already bound, overexpressed PKB could not bind and be phosphorylated on either Thr³⁰⁸ by PDK1 or on Ser⁴⁷³ by PDK2. This would also prevent its activation. Whatever the correct explanation, our data suggests that caution should be exercised in assuming that overexpressed proteins behave in the same manner as endogenous proteins. In WEHI cells, overexpression of PKB induced profound morphological changes with enhanced adherence, and these changes might themselves have altered the cellular response to TNF- α .

Any explanation of our data seems to demand that PDK2 is a distinct entity from PDK1. This was originally proposed to be the case, because PDK1 was shown not to phosphorylate Ser⁴⁷³ *in vitro* [10]. However, it was subsequently demonstrated that an interaction between the kinase domain of PDK1 and a region of protein kinase C-related kinase-2, termed PDK1-interacting fragment ('PIF'), converted it into a form that could phosphorylate both Thr³⁰⁸ and Ser⁴⁷³ [12]. The true position has since been clarified by the preparation of mouse embryonic stem cells lacking PDK1 [28]. These cells exhibit Ser⁴⁷³ phosphorylation (but not PKB activation) in response to IGF-1, confirming that there is a separate PDK2 responsible for phosphorylating Ser⁴⁷³ *in vivo*. The identity of this kinase is uncertain. Indeed, there might be more than one because both p38 kinase-dependent MAPKAPK-2 and PI3-kinase-dependent integrin-linked kinase have been implicated [13,14].

The exact roles of PKB activation by TNF- α remain to be elucidated. There is no doubt that PKB activation represents a pro-survival or anti-apoptotic signal to the cell [29]. Among other effects, it is able to inhibit caspase 9 activity by phosphorylation [30], and to phosphorylate and inactivate the pro-apoptotic protein BAD (Bcl-2/Bcl-X_L-antagonist, causing cell death) [31]. However, the interaction between PKB-dependent and -independent survival pathways in TNF- α action remains unclear. A number of studies have shown that PI3-kinase and PKB activation are required for the expression of NF- κ B-dependent genes [16,32]. However, we show here that in WEHI cells addition of wortmannin had only a modest (20%) effect on cell survival in the presence of TNF- α , unless the transcription inhibitor actinomycin D was also present (Figure 2). These data imply the presence of a transcription-dependent survival pathway that is activated by TNF- α via a mechanism largely independent of PI3-kinase activation. This has not been reported previously. When transcription was prevented, cell survival in the presence TNF- α was lowered further by wortmannin, implying the activation of an additional transcription-independent survival pathway mediated via ac-

tivation of PI3-kinase and PKB as reported by others [21]. Although the contribution of this pathway may be modest relative to a transcriptional-dependent pathway, it will respond more rapidly than the latter and might play an important role in the physiological setting. Here, the natural response of fibroblasts to TNF- α is a proliferative one that is required for wound repair following the inflammatory response. If PKB plays a role in this response, then this pathway might provide useful therapeutic targets in the future.

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