FEBS Letters 502 (2001) 84–88 FEBS 25079

p90-RSK and Akt may promote rapid phosphorylation/inactivation of glycogen synthase kinase 3 in chemoattractant-stimulated neutrophils

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Received 12 April 2001; revised 12 June 2001; accepted 2 July 2001

First published online 16 July 2001

Edited by Masayuki Miyasaka

Abstract Stimulation of neutrophils with the chemoattractant fMet-Leu-Phe (fMLP) triggers phosphorylation/inactivation of the α- and β-isoforms of glycogen synthase kinase 3 (GSK-3) with phosphorylation of the α-isoform predominating. These reactions were monitored with a phosphospecific antibody that only recognized the α- or β-isoforms of GSK-3 when these proteins were phosphorylated on serine residues 21 and 9, respectively. Inhibitor studies indicated that phosphorylation of GSK-3α may be catalyzed by the combined action of p90-RSK and Akt and may represent a new strategy by which G protein-coupled receptors inactivate GSK-3. Inactivation of GSK-3 may be one of the mechanisms that delay apoptosis in fMLP-stimulated neutrophils.

Key words: Neutrophil; Cell signaling; Chemoattractant; p90-RSK; Glycogen synthase kinase 3

1. Introduction

Resolution of inflammation requires the recognition and phagocytosis of apoptotic neutrophils by macrophages [1]. Apoptosis of neutrophils can be delayed by a variety of chemoattractants (e.g. interleukin-8, fMet-Leu-Phe (fMLP)) and other inflammatory mediators (granulocyte/macrophage colony-stimulating factor, lipopolysaccharide) through complex mechanisms that can involve phosphoinositide 3-kinase (PI 3-K), Akt or the extracellular signal-regulated kinase (ERK) pathway (e.g. [2,3]). We have recently reported that the Ser/Thr protein kinase p90-RSK-2 undergoes rapid activation in fMLP-stimulated neutrophils [4]. Complete activation of p90-RSK-2 requires the phosphorylation of this kinase on multiple sites by both autophosphorylation and the action of upstream kinases (e.g. ERK, 3-phosphoinositide-dependent protein kinase 1 (PDK 1)) [5–7]. Relatively few physiological substrates for p90-RSK-2 have been identified to date even though this kinase may have a major role in promoting cell survival [8,9].

Glycogen synthase kinase 3 (GSK-3) is a constitutively active protein kinase that participates in hierarchical phosphorylation reactions by catalyzing the phosphorylation of Ser/Thr residues that are located on the N-terminal side of a phosphoserine residue (–S/TXXXS(P)–) (e.g. [10]). Transfection of active GSK-3β into certain cells can promote spontaneous apoptosis [11] or enhance apoptosis triggered by the other agents [12]. Phosphorylation of GSK-3α on Ser-21 or GSK-3β on Ser-9 inhibits the catalytic activity of these isoforms [10,13] and thus may reduce their proapoptotic activity. Phosphorylation/inactivation of GSK-3 is catalyzed by Akt or p90-RSK in cells treated with growth factors that bind to receptor tyrosine kinases (e.g. [13,14]) or by protein kinase A (PKA) in cells stimulated with an agonist that binds to a G protein-coupled receptor [15,16]. fMLP binds to a G protein-coupled receptor on neutrophils (e.g. [17]) and triggers a myriad of biochemical events that include activation of p90-RSK-2, Akt and PKA (e.g. [4,17]). In this paper we report that neutrophils stimulated with fMLP exhibit rapid phosphorylation/inactivation of GSK-3. Inhibitor studies indicated that this reaction may be catalyzed by both p90-RSK and Akt. Thus, G protein-coupled receptors may employ a variety of pathways to promote phosphorylation/inactivation of GSK-3.

2. Materials and methods

2.1. Materials

A goat polyclonal antibody raised to a peptide corresponding to residues 722–740 of human RSK-2 (RSK-2(C-19) Ab) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Affinity-purified, rabbit polyclonal phosphospecific antibodies to GSK-3 (phospho-GSK-3α/β(Ser-21/9) Ab), Akt (phospho-Akt(Thr-308) Ab) and p90-RSK (phospho-p90-RSK(Ser-381) Ab) and a GSK-3 fusion protein were purchased from Cell Signaling Technology, Beverly, MA, USA. This GSK-3 fusion protein was a 'crosstide' of molecular mass 30 kDa that consisted of a peptide containing the residues surrounding the Ser-21/9 site in GSK-3α/β chemically attached to bacterially expressed paramyosin. Wortmannin, LY 294002 and PD 98059 were obtained from Calbiochem, San Diego, CA, USA. Protein A-4β agarose was purchased from Sigma Chemical, St. Louis, MO, USA. Sources of all other materials are described elsewhere [4,18,19].

2.2. Preparation of neutrophils

Guinea pig peritoneal neutrophils were prepared as described previously [20].

2.3. Cell stimulation and Western blotting

These procedures are described in detail in [18] and [4,19], respectively. At the end of these experiments, the blots were stripped and stained with an antibody that recognized both the phosphorylated and non-phosphorylated forms of Akt or ERK to confirm that equal amounts of protein were present in each lane of the gel (e.g. [4]).

2.4. Immunoprecipitation and assay of p90-RSK-2

Neutrophils (1.0×10^8/ml) were stimulated with 1.0 μM fMLP in a

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Abbreviations: fMLP, fMet-Leu-Phe; GSK-3, glycogen synthase kinase 3; ERK, extracellular signal-regulated kinase; PI 3-K, phosphoinositide 3-kinase; PDK-1, 3-phosphoinositide-dependent protein kinase 1; PKA, protein kinase A

0014-5793/01/$20.00
PII: S0014-5793(01)02669-2

phosphate-buffered saline medium (135 mM NaCl, 2.7 mM KCl, 16.2 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 0.90 mM $CaCl_2$ and 0.50 mM $MgCl_2$, pH 7.35) containing 7.5 mM D-glucose. At the appropriate time, the cells were rapidly lysed by transferring 0.50 ml of the reaction mixture to a microcentrifuge tube containing 2×concentrated 'immunoprecipitation buffer' (ip-B) and rapidly mixed. The final composition of ip-B was 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5.0 mM $MgCl_2$, 1.0 mM EGTA, 50 mM NaF, 10 mM sodium pyrophosphate, 1.0% (v/v) Nonidet P-40, 2.5% (v/v) glycerol, 1.0 mM Na_3VO_4 and 1.0 mM phenylmethylsulfonyl fluoride. After 30 min at 4°C, the lysates were centrifuged for 10 min at 1000×*g* to remove insoluble debris, the RSK-2(C-19) Ab (10 μg/ml) was added to the soluble fraction, and this fraction was then incubated for 2 h at 4°C. A 50% slurry of protein A Sepharose beads (60 μl) was added to the reaction mixture, which was then incubated for an additional hour at 4°C. The resulting beads containing the bound immune complexes were washed three times with ip-B (1.0 ml/wash) and three times with kinase buffer (20 mM $MgCl_2$, 1.0 mM sodium vanadate, 5.0 mM NaF, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenylphosphate and 50 mM HEPES, pH 7.4) [21]. The bound immune complexes were resuspended in 25 μl of kinase buffer containing 100 μM ATP and the GSK-3 fusion protein (10 μg/ml). Following an incubation of 30 min at 37°C, an equal volume of 2×concentrated 'solubilization buffer' was added to the reaction mixtures and the samples were boiled for 5.0 min. The final concentration of sample buffer after mixing was 2.3% (v/v) SDS, 62.5 mM Tris–HCl (pH 6.8), 5.0 mM EDTA, 10.0% (v/v) glycerol, 5.0% (v/v) 2-mercaptoethanol and 0.002% (v/v) bromophenol blue. The proteins were separated by SDS–PAGE (70 μg/ml) on 9.0% (v/v) polyacrylamide slab gels and phosphorylation of the fusion protein was monitored by Western blotting with the phospho-GSK-3α/β(Ser-21/9) Ab.

2.5. Analysis of data

Unless otherwise noted, all of the observations from the Western blots were confirmed in at least three separate experiments performed on different preparations of cells. The numbers of observations (*n*) are also based on different preparations of cells.

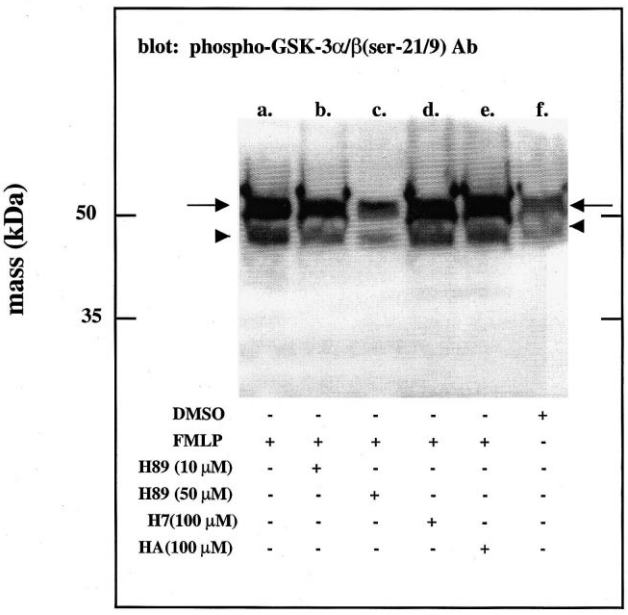

Fig. 1. Phosphorylation of GSK-3 in stimulated neutrophils. Neutrophils were stimulated with 1.0 μM fMLP for 6.0 min and phosphorylation of GSK-3 was monitored by Western blotting with a phosphospecific antibody which only recognized GSK-3α (arrow) or GSK-3β (closed arrowhead) when these isoforms were phosphorylated on Ser-21 and Ser-9, respectively. Lane a is for stimulated cells. Lane f is for unstimulated neutrophils. In certain cases (lanes b–e) the cells were treated with inhibitors for 30 min at 37°C and then stimulated with 1.0 μM fMLP for 6.0 min. Lanes b–e are for stimulated cells treated with: (b) 10 μM H89; (c) 50 μM H89; (d) 100 μM H-7 and (e) 100 μM HA1004. Conditions for cell stimulation and Western blotting are referenced in Section 2.

3. Results

3.1. Phosphorylation of GSK-3 in stimulated neutrophils

A phosphospecific antibody was utilized to monitor phosphorylation of GSK-3 in neutrophils (Fig. 1). The phospho-GSK-3α/β(Ser-21/9) Ab employed reacted with both the α- and β-isoforms of this kinase only when they were phosphorylated on Ser-21 and Ser-9, respectively. This antibody reacted with only two proteins in unstimulated (lane f) and stimulated neutrophils (lane a) and these proteins exhibited masses consistent with the α- (arrow) and β- (closed arrowhead) isoforms of GSK-3. Stimulation of neutrophils with 1.0 μM fMLP for 6.0 min resulted in an increased reactivity of these bands towards the phosphospecific antibody with the α-isoform usually predominating. The amounts of GSK-3β that reacted with the antibody were variable.

Activation of the G protein-coupled β-adrenergic receptor with adrenaline in a variety of cells leads to rapid phosphorylation of the α- and β-isoforms of GSK-3 on Ser-21 and Ser-9, respectively [15,16]. These reactions were completely blocked by an antagonist of PKA (10 μM H89) [15]. However, a variety of antagonists of PKA (10 μM H89, 100 μM H-7 or 100 μM HA1004) [15,22] did not block the increase in phosphorylation of GSK-3α on Ser-21 in fMLP-stimulated neutrophils (Fig. 1) ($n=4$). Interestingly, the GSK-3β band was partially reduced by 10 μM H89 but was largely insensitive to H-7 and HA1004. Increasing the concentration of H89 to 50 μM substantially inhibited phosphorylation of both the α- and β-isoforms of GSK-3 (Fig. 1). Recent studies have established that H89 can inhibit several protein kinases in addition to PKA [23].

3.2. Kinetics of phosphorylation of GSK-3, Akt and p90-RSK in stimulated neutrophils

Phosphospecific antibodies were utilized to monitor the phosphorylation of GSK-3, Akt and p90-RSK in neutrophils stimulated with fMLP (Fig. 2). The phosphospecific antibodies to Akt and p90-RSK only recognized these kinases when they were phosphorylated on Thr-308 and Ser-381, respectively. Activation of Akt requires phosphorylation at Thr-308 [24], whereas the role of Ser-381 in the activation of p90-RSK is complex ([6,7]; data presented below). GSK-3 underwent phosphorylation in stimulated neutrophils with maximal phosphorylation occurring at 3.0–6.0 min (Fig. 2A). In contrast, Akt underwent maximal phosphorylation within 1.0 min followed by significant dephosphorylation at 3.0–6.0 min (Fig. 2B). Interestingly, neutrophils stimulated with fMLP exhibited phosphorylation of p90-RSK with kinetics nearly identical to that of GSK-3 (Fig. 2C).

3.3. Effects of various antagonists on the phosphorylation of GSK-3

A variety of antagonists were employed to identify the protein kinase(s) that catalyze phosphorylation of GSK-3α in fMLP-stimulated neutrophils (Fig. 3). Wortmannin is an irreversible inhibitor of PI 3-K (e.g. [17]). Products of PI 3-K are required for translocation of PDK-1 to the membrane and activation of Akt in cells (e.g. [17]). Wortmannin (200 nM)

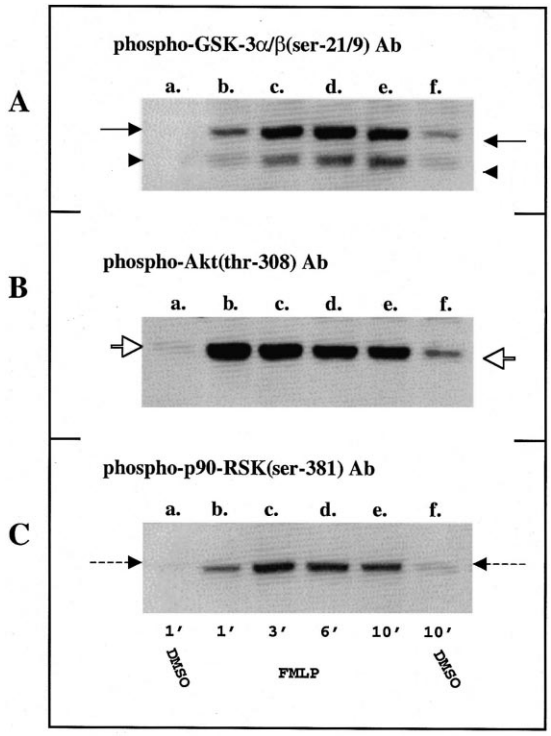

Fig. 2. Time course for the phosphorylation of GSK-3 (A), Akt (B) and p90-RSK (C) in stimulated neutrophils. Neutrophils were stimulated with 1.0 μM fMLP for the time points indicated and phosphorylation of GSK-3, Akt and p90-RSK was monitored by Western blotting with phosphospecific antibodies. The amounts of phosphorylation of these kinases in unstimulated neutrophils at 1.0 min and 10.0 min are also shown (lanes a and f). A: The antibody utilized recognized GSK-3α (arrow) and GSK-3β (closed arrowhead) only when these isoforms were phosphorylated on Ser-21 and Ser-9, respectively. B: The antibody utilized recognized Akt (open arrowhead) only when this kinase was phosphorylated on Thr-308. C: The antibody utilized recognized p90-RSK (broken arrow) only when this kinase was phosphorylated on Ser-381. Conditions for cell stimulation and Western blotting are referenced in Section 2.

completely blocked phosphorylation of Akt on Thr-308 (Fig. 3B, c1 and c2) but did not affect phosphorylation on GSK-3α (Fig. 3A, c1 and c2). Compound PD 98059 blocks activation of p90-RSK in neutrophils [4] and other cell types by blocking activation of MEK and ERK [25]. PD 98059 (50 μM) partially reduced phosphorylation at both Ser-381 in p90-RSK (Fig. 3C, d) and Ser-21 in GSK-3α (Fig. 3A, d) (see below). However, the combination of wortmannin (200 nM) plus PD 98059 (50 μM) produced a striking reduction in phosphorylation of GSK-3α (Fig. 3A, e). The decreases in phosphorylation of GSK-3α were estimated by densitometry by comparing the peak height of the band in lane b with that in lanes c, d and e. Treatment of neutrophils with 200 nM wortmannin, 50 μM PD 98059 and 200 nM wortmannin plus 50 μM PD 98059 reduced the content of phosphate at Ser-21 in GSK-3α by 11 ± 2%, 37 ± 9% and 85 ± 7% (S.D., $n = 3$), respectively. As noted above, the reactivity of GSK-3β towards the phospho-GSK-3α/β(Ser-21/9) Ab was variable. However, when significant amounts of GSK-3β were observed it behaved similarly to GSK-3α in terms of its sensitivity to wortmannin and/or PD 98059 (Fig. 3A).

In contrast to wortmannin, compound LY 294002 is a reversible inhibitor of PI 3-K that competes for the ATP binding site on this lipid kinase [26]. The effects of LY 294002 on the phosphorylation of GSK-3 in fMLP-stimulated neutrophils are presented in Fig. 4. Treatment of neutrophils with 50 μM LY 294002 or 50 μM LY 294002 plus 50 μM PD 98059 reduced the content of phosphate at Ser-21 in GSK-3α by 30 ± 9% and 97 ± 5% (S.D., $n = 3$), respectively. Treatment of neutrophils with 50 μM LY 294002 alone for 30 min at 37°C followed by stimulation of the cells with 1.0 μM fMLP for 6.0 min reduced phosphorylation of Akt and p90-RSK by 100 ± 0% and 18 ± 15% (S.D., $n = 3$), respectively (data not shown). Thus, two structurally and mechanistically distinct antagonists of PI 3-K were each capable of substantially inhibiting the phosphorylation of GSK-3α in stimulated neutrophils when combined with PD 98059.

Wortmannin and PD 98059 blocked phosphorylation at Ser-381 in p90-RSK in a manner similar to that described above for GSK-3 (Fig. 3C). Treatment of neutrophils with 200 nM wortmannin, 50 μM PD 98059 and 200 nM wortmannin plus 50 μM PD 98059 reduced the content of phosphate at Ser-381 in p90-RSK by 0%, 46 ± 2% and 63 ± 3% (S.D. or range, $n = 2$–3), respectively. The concentrations of inhibitors and conditions used in these studies did not affect cell viability, as measured by the exclusion of trypan blue or by the release of lactate dehydrogenase from the cells (data not shown [4,27]).

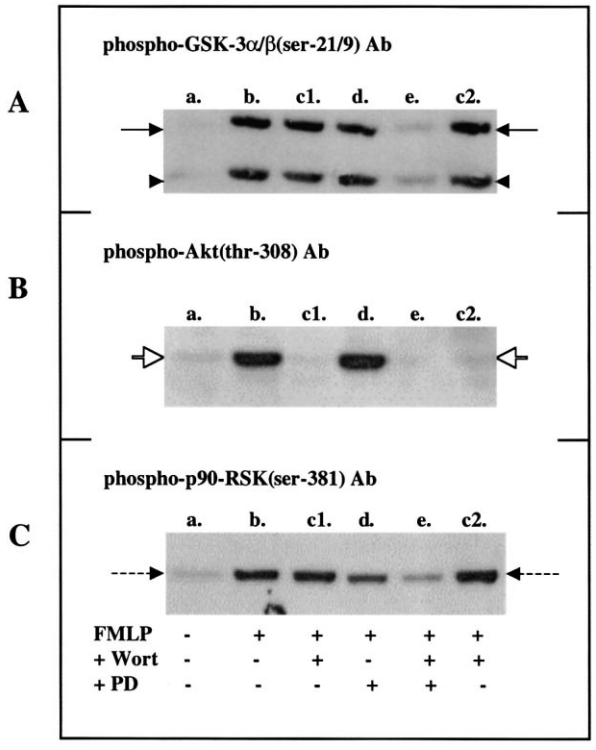

Fig. 3. Effects of different antagonists on the phosphorylation of GSK-3 (A), Akt (B) and p90-RSK (C) in stimulated neutrophils. Cells were treated with the inhibitors for 30 min at 37°C and then stimulated with 1.0 μM fMLP for 6.0 min. Lane a is for unstimulated cells. Lane b is for stimulated cells. Lanes c–e are for stimulated cells treated with: (c1 and c2; two different samples) 200 nM wortmannin; (d) 50 μM PD 98059 and (e) 50 μM PD 98059 plus 200 nM wortmannin. Site-specific phosphorylation of GSK-3, Akt and p90-RSK was monitored with the phosphospecific antibodies described in the legend to Fig. 2. Conditions for cell stimulation and Western blotting are referenced in Section 2.

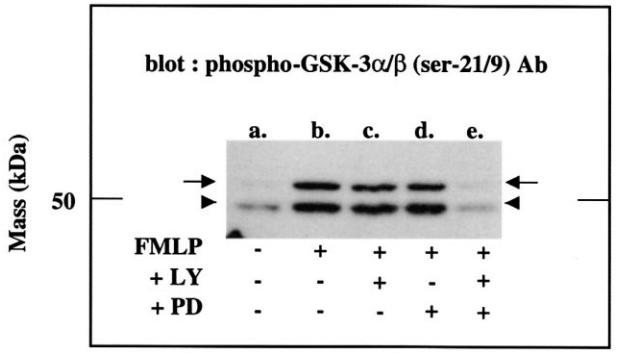

Fig. 4. Effects of LY 294002 and PD 98059 on the phosphorylation of GSK-3 in stimulated neutrophils. Cells were treated with the inhibitors for 30 min at 37°C and then stimulated with 1.0 μM fMLP for 6.0 min. Lane a is for unstimulated cells. Lane b is for stimulated cells. Lanes c–e are for stimulated cells treated with: (c) 50 μM LY 294002, (d) 50 μM PD 98059 and (e) 50 μM LY 294002 plus 50 μM PD 98059. Site-specific phosphorylation of GSK-3 was monitored with the phospho-GSK-3α/β(Ser-21/9) Ab. Conditions for cell stimulation and Western blotting are referenced in Section 2.

3.4. *Effects of wortmannin and PD 98059 on the activation of p90-RSK-2*

Studies were undertaken to determine if p90-RSK-2 from stimulated neutrophils exhibited enhanced activity towards the Ser-21/9 sites in GSK-3α/β and if so whether this activation was reduced by PD 98059 and/or wortmannin (Fig. 5). p90-RSK-2 was immunoprecipitated from neutrophil lysates, assayed with a fusion protein containing the Ser-21/9 site in GSK-3α/β and phosphorylation of this substrate was monitored with the phospho-GSK-3α/β(Ser-21/9) Ab. p90-RSK-2 from fMLP-stimulated neutrophils exhibited substantial activity with this substrate. In contrast, little activity was observed with immunoprecipitates from unstimulated cells or if non-immune serum was used in place of the RSK-2(C-19) Ab (Fig. 5A).

The amounts of activity for p90-RSK-2 in the immune complexes were markedly reduced if the cells were treated with PD 98059 (50 μM) for 15 min before stimulation with fMLP (Fig. 5B, c). In contrast, wortmannin (200 nM) alone did not block activation of p90-RSK-2 (Fig. 5B, d) and the combination of wortmannin (200 nM) plus PD 98059 (50 μM) was only slightly more effective than PD 98059 alone (Fig. 5B, e). Treatment of neutrophils with PD 98059 (50 μM), wortmannin (200 nM) or PD 98059 (50 μM) plus wortmannin (200 nM) reduced the amount of phosphate in the fusion protein by 84 ± 13%, 8 ± 11% and 87 ± 8% (S.D., $n = 3$), respectively. These data demonstrate that the activity of p90-RSK-2 towards the Ser-21/9 site in GSK-3α/β is markedly increased in stimulated neutrophils and that this increase is due largely to the MEK/ERK pathway and not to PDK-1 or Akt.

4. Discussion

In this communication we report that GSK-3α undergoes rapid phosphorylation on Ser-21 in stimulated neutrophils. A variable amount of phosphorylation of GSK-3β on Ser-9 was also observed. Further, we provide evidence that these phosphorylation events may be catalyzed by both p90-RSK-2 and Akt in fMLP-stimulated neutrophils. In particular, antagonists which block activation of p90-RSK-2 and Akt inhibited phosphorylation of GSK-3α in a synergistic manner (Figs. 3 and 4). In addition, the progress curve for phosphorylation/activation of Akt was consistent with this kinase being located upstream of GSK-3 whereas that for p90-RSK-2 was consistent with this kinase operating either upstream or parallel to GSK-3 (Fig. 2) [4]. As noted above, p90-RSK-2 and Akt

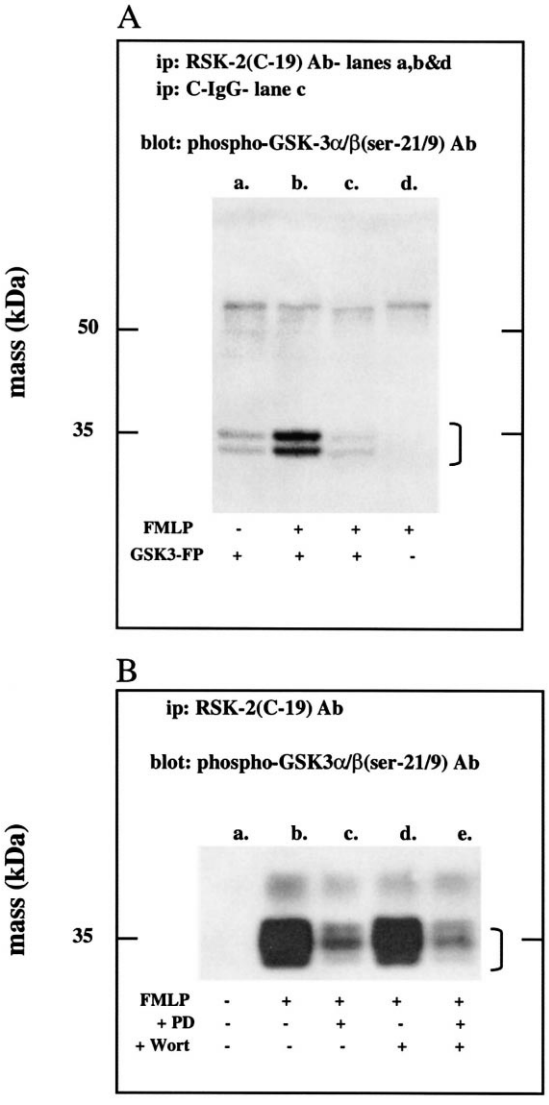

Fig. 5. Ability of p90-RSK-2 from stimulated neutrophils to catalyze phosphorylation of the Ser-21/9 site in a GSK-3 fusion protein. Effects of PD 98059 and wortmannin on activation of p90-RSK-2. A: p90-RSK was immunoprecipitated from neutrophil lysates (lanes a, b and d) and assayed for its ability to catalyze phosphorylation of a GSK-3 fusion protein. Lane c is for neutrophil lysates treated with non-immune serum (C-IgG) in place of the p90-RSK-2(C-19) Ab. Cells were treated for 6.0 min with 0.25% (v/v) dimethyl sulfoxide (lane a; unstimulated neutrophils) or 1.0 μM fMLP (lanes b–d) before preparing the lysates. B: Neutrophils were treated with different inhibitors for 30 min at 37°C, stimulated with 1.0 μM fMLP for 6.0 min and then lysed. p90-RSK-2 was immunoprecipitated from the lysates and assayed with the GSK-3 fusion protein as the substrate. Lane a is for unstimulated cells. Lane b is for stimulated cells. Lanes c–e are for stimulated cells treated with: (c) 50 μM PD 98059, (d) 200 nM wortmannin and (e) 50 μM PD 98059 plus 200 nM wortmannin. Conditions for cell stimulation, immunoprecipitation and assaying kinase activity in immune complexes are described/referenced in Section 2.

catalyze the phosphorylation of GSK-3α on Ser-21 in vitro [13,14].

PD 98059 and wortmannin were recently shown to have the most impressive selectivity when a variety of inhibitors were compared against a large panel of protein kinases [23]. However, it should be emphasized that these antagonists block activation of Akt and p90-RSK indirectly, through inhibition of upstream components (e.g. [17,25]). Thus, the possibility exists that these antagonists may also be affecting unknown kinases that are also active against GSK-3. It is not possible to employ the techniques of molecular biology to directly investigate the involvement of p90-RSK-2 and Akt in the phosphorylation/inactivation of GSK-3 in primary neutrophils because these cells are short-lived. Immunohistochemical studies may be of value here to show that these kinases are co-localized at the appropriate time points after cell stimulation.

Compound PD 98059 and wortmannin also synergistically inhibited phosphorylation of p90-RSK on Ser-381 in stimulated neutrophils (Fig. 3C, e). However, phosphorylation at this site does not appear to be closely associated with the activity of p90-RSK. In particular, PD 98059 alone blocked activation of p90-RSK-2 by 84 ± 13% (Fig. 5B, c) but inhibited phosphorylation at Ser-381 by only 46 ± 2% (Fig. 3C, d). Thus, it is unlikely that the synergistic inhibition of GSK-3 phosphorylation by PD 98059 and wortmannin can be explained simply by the effects of these compounds on p90-RSK-2 alone.

p90-RSK-2 is a highly unusual kinase in that it contains two distinct active sites/protein kinase domains that reside in the N-terminal and C-terminal regions of the protein [5]. The N-terminal kinase domain (NTD) catalyzes the phosphorylation of exogenous substrates whereas the C-terminal domain (CTD) is required for complete activation of the NTD [5,6]. The mechanism of activation of p90-RSK-2 is complex and involves phosphorylation on Ser-381 and several other residues [6]. Phosphorylation at Ser-381 can be catalyzed by the CTD of the kinase itself but only after activation of this domain by an ERK- or PDK-1-catalyzed phosphorylation event [6,7]. The ability of PD 98059 and wortmannin to synergistically inhibit phosphorylation on Ser-381 suggests that both of these reactions for activating the CTD may be operating in fMLP-stimulated neutrophils and that products of PI 3-K may participate in the PDK-1-dependent reaction(s).

As noted above, transfection studies indicate that GSK-3β can trigger or enhance apoptosis in various cell types [11,12]. Whether GSK-3α or GSK-3β performs a similar function in neutrophils is not yet known. Both p90-RSK and Akt have substrates in addition to GSK-3 that influence apoptosis [8,9,28]. In fact, either LY 294002 or PD 98059 alone can prevent the delay in neutrophil apoptosis triggered by chemoattractants [2,3]. Thus, these antagonists are not likely to be helpful in determining if GSK-3 is involved in apoptosis in neutrophils. However, the recent availability of selective antagonists of GSK-3 [29] may prove useful in addressing this question.

Acknowledgements: These studies were supported by Grants DK 50015, AI 23323, PO1 DE 13499 and KO8 NS 01922 (to L.C.) from the National Institutes of Health.

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