Initiation factor eIF2B not p70 S6 kinase is involved in the activation of the PI-3K signalling pathway induced by the v-*src* oncogene

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Abstract Our data show that in hamster fibroblasts transformed by Rous sarcoma virus (RSV), the phosphoinositide 3′-kinase (PI-3K)/Akt/glycogen synthase kinase 3 antiapoptotic pathway is upregulated and involved in increased protein synthesis through activation of initiation factor eIF2B. Upon inhibition of PI-3K by wortmannin, phosphorylation of 70-kDa ribosomal protein S6 kinase (p70 S6k) and its physiological substrate, ribosomal protein S6, decreased in the non-transformed cells but not in RSV-transformed cells. Thus PI-3K, which is thought to be involved in regulation of p70 S6k, signals to p70 S6k in normal fibroblasts, but it does not appear to be an upstream effector of p70 S6k in fibroblasts transformed by v-*src* oncogene, suggesting that changes in the PI-3K signalling pathway upstream of p70 S6k are induced by RSV transformation.

Key words: Phosphoinositide 3′-kinase signalling pathway; mRNA translation; 70-kDa Ribosomal protein S6 kinase; v-Src transformation; Initiation factor eIF2B

1. Introduction

Several proteins which undergo control in mammalian translational machinery are targets of the mammalian target of rapamycin (mTOR). Among them is the 70-kDa ribosomal protein S6 kinase (p70 S6k) that catalyzes phosphorylation of the 40S ribosomal protein S6. Activation of p70 S6k leads to the upregulation of protein biosynthesis and increases the translational capacity of the cell by stimulation of the selective translation of 5′-TOP (terminal oligopyrimidine tract) mRNAs that primarily encode proteins and components of the translational apparatus. Recruitment of these mRNAs to translating polysomes may be the mechanism by which p70 S6k regulates cell growth [1].

Expression of a specific subset of mRNAs that possess long highly structured 5′-untranslated regions is also regulated at the level of translation initiation by rapamycin-sensitive mechanisms. Rapamycin inhibits cap-dependent translation probably by blocking the function of the cap-binding protein eIF4E which is regulated by the translation repressor proteins (4E-BP1,2) [2]. By controlling cap-dependent translation and the translation of 5′-TOP mRNA, the mTOR signalling pathway upregulates the translational machinery under favorable growth conditions [3].

Another important signalling pathway that is activated by growth-promoting stimuli is the rapamycin-insensitive pathway which involves phosphoinositide 3′-kinase (PI-3K). This pathway is inhibited by wortmannin, a selective inhibitor of PI-3K. Activation of the PI-3K pathway is initiated by an increased level of specific 3-phosphoinositides (3-PtdIns) that are generated by the activated PI-3K. Interactions of these lipids with the lipid-binding 'pleckstrin homology' (PH) domains present in some proteins leads to the activation of serine/threonine protein kinases including 3′-phosphoinositide-dependent protein kinase 1, protein kinase B (PKB/Akt) and p70 S6k, and inactivation of glycogen synthase kinase 3 (GSK-3) [4]. Nevertheless, the regulation of p70 S6k involving inputs from signalling events linked to PI-3K is not yet fully understood. Recent work suggests that this may involve the components of the tuberous sclerosis complex TSC1 and TSC2, TSC2 being a direct substrate for Akt [5].

Since the activated protein product of the *src* gene is involved in multiple mechanisms by which cells are transformed into the malignant phenotype, we analyzed cells that were transformed by Rous sarcoma virus (RSV) to explore the mechanisms that control mRNA translation and may be involved in the process of cell malignant transformation induced by the v-*src* oncogene. Significantly higher amounts of the activated Src protein are present in RSV-transformed fibroblasts compared to non-transformed hamster fibroblasts [6]. The enhanced expression of Src correlated with increased levels of overall protein synthesis in these cells and, upon rapamycin treatment, both synthesis of Src and global protein synthesis were less suppressed in RSV-transformed cells relative to control cells, indicating that some other rapamycin-insensitive regulatory mechanisms are activated in hamster fibroblasts by their RSV transformation [7]. To identify such regulatory mechanisms, we analyzed PI-3K signalling and found that the PI-3K signalling pathway differentially controls translation in normal and RSV-transformed hamster fibroblasts.

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Abbreviations: RSV, Rous sarcoma virus; PI-3K, phosphoinositide 3′-kinase; mTOR, mammalian target of rapamycin; eIF, eukaryotic initiation factor; 4E-BP1, eukaryotic initiation factor 4E-binding protein; GSK-3, glycogen synthase kinase 3; p70 S6k, 70-kDa ribosomal protein S6 kinase

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2. Materials and methods

2.1. Materials

[γ-^{32}P]ATP (3000 Ci/mmol), [^{35}S]methionine/cysteine (Promix), [^{3}H]GDP (11.9 Ci/mmol), second antibody (anti-rabbit Ig and anti-mouse Ig), glutathione-Sepharose 4B and ECL Western blotting detection kits were from Amersham, UK. Wortmannin, rapamycin and all general reagents were obtained from Sigma (USA), unless otherwise stated. Microcystin LR was from Calbiochem (USA) and TLC plates SilicaGel-Kieselgur were obtained from Merck (Germany). Polyclonal antibody anti-p70 S6k, monoclonal anti-phosphotyrosine antibody PY 99 and anti-4E-BP1 antibody were obtained from Santa Cruz Biotechnology (USA). Monoclonal anti-Src antibody LA 074 were from Quality Biotech (USA). Polyclonal antibodies anti-Akt, anti-phospho-Akt(Ser473), anti-phospho-S6 ribosomal protein(Ser235/236) and the GSK-3 fusion protein were from Cell Signalling Technology (USA) and anti-GSK-3β antibody was obtained from Transduction Laboratories.

2.2. Cell culture and preparation of cell extracts

The hamster tumor cell line H19 and the control hamster embryo cell line NIL-2 were grown as described in [7]. Cells were incubated with rapamycin as described [7] or incubated with wortmannin added to a final concentration of 100 nM (dimethyl sulfoxide (DMSO) solution diluted in medium 1×10^{6}) for 10 min or with DMSO carrier alone for 10 min, unless otherwise indicated. The cells were then lysed in ice-cold extraction buffer A (40 mM β-glycerophosphate, 100 mM HEPES pH 7.4, 5 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% Triton X-100, 0.05 μM microcystin and the proteinase inhibitors pepstatin, antipain and leupeptin (0.04 μg/ml each)) unless otherwise indicated. Measurement of protein synthesis was performed as described previously [7] with the exception that the cells were metabolically labelled in a medium containing [^{35}S]methionine/cysteine (100 μCi/ml), and wortmannin (100 nM final concentration in medium) instead of rapamycin, was used. To determine the expression of the Src protein, the extracts of the labelled cells (0.5 mg) that were non-treated or treated with 100 nM wortmannin for 20 h were immunoprecipitated, subjected to electrophoresis followed by immunoblotting and radioactivity associated with the protein band corresponding to Src was measured as described in [7].

2.3. Immunoprecipitation of proteins

Proteins (0.6 mg) were immunoprecipitated from the cell extract by incubation for 2 h at 4°C either with specific monoclonal antibodies preadsorbed to 10 μl protein G-Sepharose or with specific polyclonal antibodies preadsorbed to 2.5 mg protein A-Sepharose. Immune complexes were washed with ice-cold extraction buffer and the proteins were either eluted from the beads by boiling in electrophoresis sample buffer or resuspended in 50 mM Tris–HCl pH 7.5 to a final volume of 10 μl.

2.4. Protein kinase assays

2.4.1. PKB/Akt activity. Peptide and protein phosphorylation assays of PKB/Akt activity were performed in a final volume of 20 μl as described in [8]. For the RPRAATF peptide phosphorylation assay, the reaction mixture (20 μl) was spotted onto P81 filter paper followed by several washes with 75 mM phosphoric acid and the radioactivity associated with the dried filters was counted by liquid scintillation spectrometry. When the protein substrate GSK-3 was used, incubation of the reaction mixtures was terminated by the addition of electrophoretic sample buffer and the samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Gels were then stained, washed in neutral solution, dried and autoradiographed as in [9].

2.4.2. GSK-3 activity. The assay was carried out in 25 μl final volume of the mixture containing 50 mM Tris–HCl pH 7.5, 10 μl immunoprecipitate, 50 μM substrate peptide RRAAEELDSRAGpSPQ [10,11], 1 mM dithiothreitol (DTT), 10 mM $MgCl_2$, 100 μM ATP and 2 μCi [γ-^{32}P]ATP. The mixtures were incubated for 7 min at 30°C. 20 μl of the reaction mixtures were spotted on P81 filters and treated as described above.

2.5. Thin layer chromatography (TLC) of the PI-3K lipid products

Cells were extracted in lysis buffer containing 1% Triton X-100, 40 mM β-glycerophosphate, 5 mM DTT. Protein extract (2 mg) was immunoprecipitated overnight at 4°C with 10 μl anti-phosphotyrosine antibody. Phosphatidylinositol was dispersed by sonication in a buffer containing 20 mM Tris–HCl pH 7.5, 1 mM EDTA and 5 mM DTT and the immunoprecipitates were preincubated with the lipid solution at room temperature for 10 min. The reaction mixture (50 μl) contained 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 5 mM DTT, 10 mM $MgCl_2$, 25 μg of phosphatidylinositol, 100 μM ATP and 2 μCi [γ-^{32}P]ATP. The reaction was carried out for 10 min at 37°C and terminated by the addition of 200 μl 1 N HCl, 80 μl methanol and 80 μl chloroform. After centrifugation, the upper phase was removed and the chloroform phase was washed twice with 200 μl mixture containing 1 N HCl:methanol (1:1). 30 μl of the chloroform extract was spotted on an oxalate-treated SilicaGel-Kieselgur TLC plate that was developed in a mobile phase of *n*-propanol:2 M acetic acid (65:35) [12], air-dried and quantified by InstantImager autoradiography (Packard).

2.6. Initiation factors eIF2 and eIF2B

For analysis of initiation factors eIF2B and eIF2, lysis buffer G (50 mM HEPES pH 7.4, 0.2 mM EGTA, 0.2 mM EDTA, 10% glycerol (v/v), 1% Triton X-100, 0.2 mM benzamidine, 100 mM KCl, 0.5 mM PMSF, 1 μM microcystin, pepstatin, antipain and leupeptin (0.4 μg/ml each), 1 mM DTT) was used. eIF2 was purified from hamster fibroblasts NIL-2 principally as described in [13]. The phosphorylation state of eIF2α was examined by isoelectric focusing of the partially purified eIF2α as described [14].

2.7. eIF2B activity measurement

A binary complex eIF2–[^{3}H]GDP was formed as described [15]. Briefly, 1 pmol eIF2 was incubated with 0.5 μM [^{3}H]GDP in 20 mM Tris–HCl, pH 7.6, 100 mM KCl, 1% bovine serum albumin, and 1 mM DTT. After incubation at 30°C for 15 min, 5 mM $MgCl_2$ and 1 mM GTP were added. Cell extracts (10 μl) were added to eIF2–[^{3}H]GDP complexes and incubated for another 5 min at 30°C. The [^{3}H]GDP–GTP exchange reaction was terminated by adding a cold wash buffer containing 50 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 100 mM KCl. The mixture was filtered through nitrocellulose filters and the activity of eIF2B was determined by quantification of the amount of eIF2–[^{3}H]GDP retained on the filter.

2.8. Statistical analysis

The data are expressed as the mean of at least three independent experiments performed in duplicate ± S.D. The significance of the experimental data was validated using Student's *t*-test and differences were considered to be significant when $P<0.05$.

3. Results

3.1. Effect of wortmannin on global and Src protein synthesis

Two cell lines were used to study changes in intracellular signalling induced by RSV transformation: control, non-transformed hamster fibroblasts NIL-2 and RSV-transformed hamster fibroblasts (H19 cells). As we have previously reported the enhanced overall protein synthesis in H19 cells was partially decreased by rapamycin [7]. Here we found that upon wortmannin treatment at nanomolar concentrations, which is known to selectively inhibit PI-3K, global protein synthesis was inhibited less in the control NIL-2 cells (by 12.3 ± 0.4%) than in RSV-transformed H19 cells (by 21.5 ± 0.8%; $P<0.002$), indicating a greater requirement for PI-3K activity in regulation of protein biosynthesis in fibroblasts transformed by v-*src* oncogene than in the non-transformed fibroblasts.

In addition, treatment of cells with 100 nM wortmannin for 20 h resulted in a greater decrease in expression of Src protein in v-*src*-transformed H19 cells (by 20 ± 1.8%) than in the control NIL-2 cells (by 14 ± 1.0%; $P<0.002$), suggesting an involvement of PI-3K signalling in Src protein synthesis, which is increased in RSV-transformed cells [6,7].

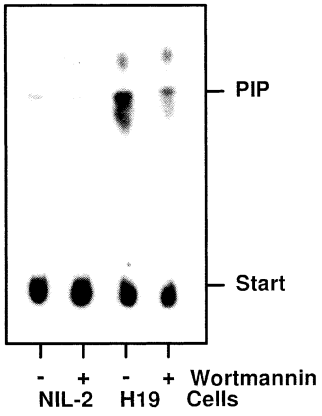

Fig. 1. Phosphotyrosine-associated PI-3K activity. Chloroform extracts of the reaction mixtures were subjected to TLC following the incubation of phosphatidylinositol with [γ-^{32}P]ATP and PI-3K present in anti-phosphotyrosine immunoprecipitates from control (NIL-2) and RSV-transformed (H19) cells that were either non-treated or treated with wortmannin as described in Section 2. The position of phosphatidylinositol phosphate on the autoradiograph is indicated.

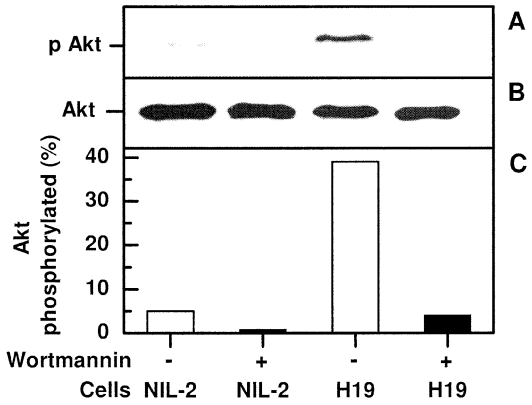

Fig. 2. Examination of the phosphorylation state of Akt in hamster fibroblasts. NIL-2 and H19 cells were non-treated or treated with wortmannin as described in Section 2. Crude cell extracts (20 μg) were analyzed by SDS–PAGE followed by Western blotting using: (A) anti-phospho-Akt(Ser473) antibody and (B) anti-Akt antibody. C: Densitometric analysis (ImageQuant, Molecular dynamics, Bio-Rad) of the Western blot of the phosphorylated Akt (A). Data are expressed as relative phosphorylation of Akt (A) to the signal for Akt (B).

3.2. Activity of PI-3K and PKB/Akt

PI-3K is highly activated in RSV-transformed hamster fibroblasts as shown by TLC analysis of the lipid products generated by phosphotyrosine-associated PI-3K. Almost four-fold higher amounts of PtdIns were produced in vitro by PI-3K immunoprecipitated from H19 cells compared to PtdIns generated by PI-3K immunoprecipitated from NIL-2 cells (Fig. 1). Wortmannin treatment decreased the amounts of PtdIns produced by PI-3K by 66% in RSV-transformed cells.

The lipid products of PI-3K are known to bind to the PH domain of Akt/PKB, and in addition to lipid binding, phosphorylation of Ser473 and Thr308 is required for activation of Akt [16]. In RSV-transformed cells, Ser473 in Akt is highly phosphorylated as seen in the Western blots of the cell extracts detected by antibodies that specifically recognized Akt phosphorylated at Ser473 (Fig. 2). This phosphorylation was blocked by wortmannin, confirming a requirement for PI-3K for Akt phosphorylation.

To demonstrate activation of Akt, we performed direct kinase assays on Akt present in H19 and NIL-2 cells, using the synthetic peptide RPRAATF, which is a relatively specific substrate for Akt [8,17]. As seen in Fig. 3A, phosphorylation of the peptide was highly increased when it was catalyzed by Akt present in RSV-transformed cells compared to phosphorylation catalyzed by Akt from the normal cells and was blocked upon wortmannin treatment, indicating that PI-3K is involved in the activation of Akt in H19 cells.

Also a GSK-3β fusion protein was used as a substrate to assay Akt. The data confirm that the wortmannin-sensitive activity of Akt immunoprecipitated from H19 cells is markedly enhanced compared to the activity of Akt immunoprecipitated from NIL-2 cells (Fig. 3B).

3.3. Involvement of p70 S6k in PI-3K signalling

Akt may lie upstream of p70 S6k which is thought to be controlled by both the rapamycin-sensitive signalling pathway of mTOR and the wortmannin-sensitive PI-3K signalling pathway. When the state of activation of p70 S6k was analyzed by means of its migration on SDS–PAGE (increased phosphorylation and thus activation of p70 S6k is manifested as lower electrophoretic mobility on SDS–PAGE), four bands corresponding to p70 S6k could be distinguished on SDS–PAGE (Fig. 4A). An apparently highly phosphorylated form of p70 S6k, represented by the most slowly migrating protein band δ, was present in H19 cells, indicating that p70 S6k was clearly more highly phosphorylated (and therefore activated) in RSV-transformed H19 cells than in the NIL-2 cells. While treatment of cells with rapamycin resulted in dephosphorylation of p70 S6k in both cell lines, NIL-2 and H19 [7], treatment of cells with wortmannin decreased the phosphorylation of p70 S6k only in NIL-2 cells. Protein band α, the least phosphorylated form of p70 S6k, was present in wortman-

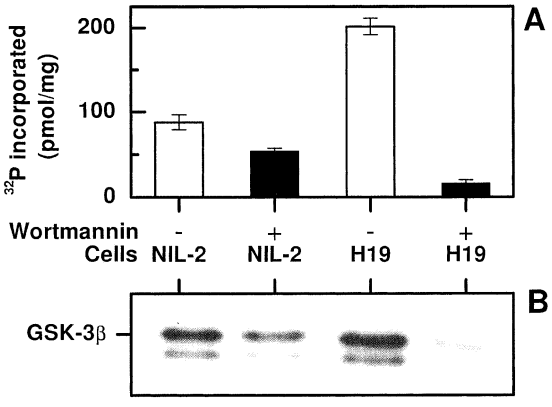

Fig. 3. PI-3K-dependent activity of Akt/PKB. Control (NIL-2) and RSV-transformed (H19) cells, which either were non-treated or were treated with wortmannin, were assayed in vitro for Akt protein kinase activity. A: RPRAATF peptide (150 μg) was used as a substrate in reaction mixtures containing crude cell extracts (5 μg) as described in [8]. Data are the mean of triplicate determination of three separate experiments ± S.D. B: Autoradiography of SDS–PAGE (15% gel) of the reaction mixtures containing Akt immunoprecipitated from NIL-2 and H19 cells, following their incubation in the presence of [γ-^{32}P]ATP and 1 μg GSK-3β fusion protein as described in Section 2.

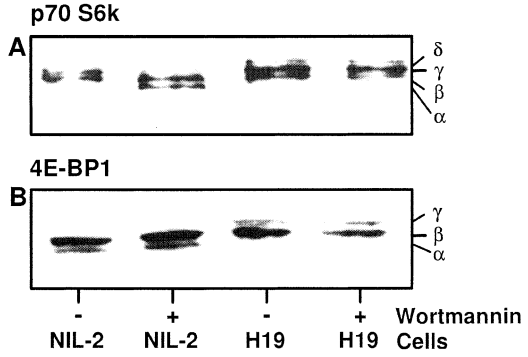

Fig. 4. Effect of wortmannin on the phosphorylation state of p70 S6k and 4E-BP1 in hamster fibroblasts. Symbols indicate different phosphorylated forms of the analyzed proteins. A: Phosphorylation of p70 S6k. Crude cell extracts of the control cells NIL-2 and RSV-transformed cells H19 were analyzed by SDS–PAGE followed by Western blotting using anti-p70 S6k antibody. Western blot from the SDS–PA gels of the extracts (15 μg) of the cells that either were non-treated or were treated with 100 nM wortmannin as described in Section 2. B: Phosphorylation of 4E-BP1. Cell extracts of control NIL-2 and RSV-transformed H19 cells, which either were non-treated or were treated with 100 nM wortmannin as described in Section 2, were subjected to precipitation and then analyzed by SDS–PAGE followed by Western blotting as described previously [7].

nin-treated control cells, but not in RSV-transformed cells whether treated with wortmannin or not, demonstrating that the level of phosphorylation of p70 S6k was unchanged upon wortmannin treatment of the transformed cells (Fig. 4A).

The translational repressor protein 4E-BP1 is also regulated by the mTOR pathway. In order to assess the effect of wortmannin on its state of phosphorylation, which is increased in RSV-transformed H19 cells [7], we analyzed trichloroacetic acid precipitates of the crude extracts of H19 and NIL-2 cells. As shown in Fig. 4B, three bands corresponding to 4E-BP1 could be distinguished on SDS–PAGE. The most slowly migrating protein band γ, representing an apparently highly phosphorylated form of 4E-BP1, was present in H19 cells. Protein band α with the highest electrophoretic mobility, the least phosphorylated form of 4E-BP1, was present in the control cells, but not in the transformed cells. As demonstrated, the level of 4E-BP1 phosphorylation was unchanged upon wortmannin treatment of both the control and RSV-transformed hamster fibroblasts.

These findings show that the increased PI-3K activity in hamster fibroblasts induced by v-*src* transformation is not involved in the upregulation of the phosphorylation of p70 S6k and 4E-BP1 in the H19 cells.

In agreement with these data phosphorylation of the ribosomal S6 protein, the substrate of p70 S6k, was found to be greatly reduced upon rapamycin treatment in the non-transformed NIL-2 cells and in RSV-transformed H19 cells, while treatment of the cells with wortmannin resulted in a decrease in S6 phosphorylation only in the normal NIL-2 cells (by 60 ± 1.1%) but not in RSV-transformed cells (Fig. 5). This suggests that RSV transformation of hamster fibroblasts changes upstream signalling to p70 S6k to render it independent of the PI-3K pathway.

3.4. GSK-3 activity

To examine further PI-3K signalling in hamster fibroblasts, we examined the activity of GSK-3, a physiological substrate for Akt [18]. As shown in Fig. 6, transformation of cells by the v-*src* oncogene induced a 43% decrease in the ability of GSK-3β immunoprecipitates to phosphorylate the peptide RRAAEELDSRAGS(P)PQ, reproducing a phosphoacceptor site in the molecule of translation initiation factor eIF2B [10] that is a GSK-3 substrate. RSV-induced GSK-3 inactivation was blocked by wortmannin, indicating the upstream regulation of GSK-3 by PI-3K. Inhibition of PI-3K evidently alleviated the inhibition of GSK-3. This confirms that Akt activation is dependent on PI-3K signalling in H19 cells.

3.5. eIF2B activity

Phosphorylation of eIF2B by GSK3 results in its inactivation [14,15]. We therefore examined the GDP/GTP exchange activity of eIF2B in the non-transformed and RSV-transformed cells and found that the activity of eIF2B increased in hamster fibroblasts upon v-*src*-transformation (Fig. 7). Wortmannin reduced the enhanced activity of eIF2B in RSV-transformed H19 cells, and had no effect on the control NIL-2 cells.

Since the increased eIF2B activity in RSV-transformed cells could in principle reflect decreased levels of the physiological

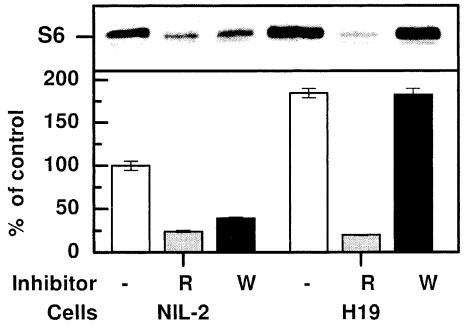

Fig. 5. Analysis of the phosphorylation of S6 ribosomal protein in NIL-2 and H19 cells. Cells were non-treated or treated with 20 nM rapamycin (R) or 100 nM wortmannin (W) for 30 min and 10 min before lysis, respectively, as described in Section 2. A: Crude cell extracts (20 μg) of the control NIL-2 cells and RSV-transformed cells H19 were analyzed by SDS–PAGE followed by Western blotting using anti-phospho-S6 antibody. B: Densitometric analysis (ImageQuant, Molecular dynamics, Bio-Rad) of the Western blot of the phosphorylated S6 protein (A). Data are expressed as a mean of four separate experiments ± S.D.

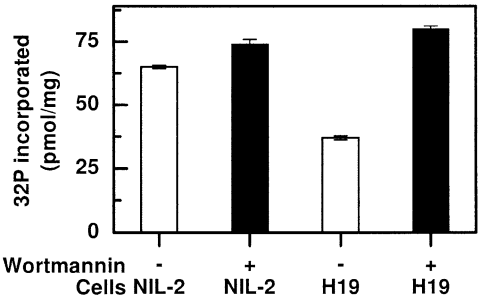

Fig. 6. Activity of GSK-3β in hamster fibroblasts. In vitro phosphorylation of the substrate peptide RRAAEELDSRAGS(P)PQ was assayed following the immunoprecipitation of GSK-3β from the cell extracts of the control (NIL-2) and RSV-transformed (H19) cells with anti-GSK-3β antibody as described in Section 2. The cells were either non-treated or treated with 100 nM wortmannin as indicated. Data are the mean of five separate experiments ± S.D.

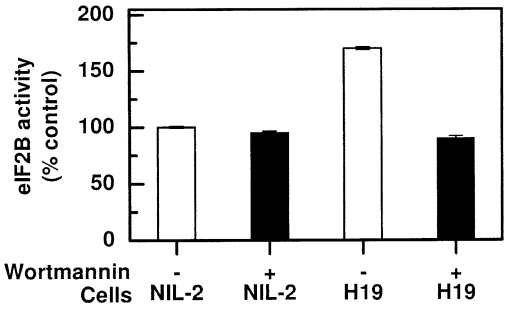

Fig. 7. GDP/GTP exchange activity of initiation factor eIF2B. The activity of eIF2B was measured in the extracts from analyzed cells non-treated or treated with 100 nM wortmannin for 10 min before lysis. Initiation factor eIF2, the substrate for eIF2B, was purified from NIL-2 cells as described in Section 2. Activity of eIF2B was expressed as a percentage of [^{3}H]GDP released from the eIF2–[^{3}H]GDP complex. eIF2B activity corresponding to control 2.1 ± 0.6 pmol was considered 100%. Data represent the mean of three individual experiments ± S.D.

inhibitor, phosphorylated eIF2α (eIF2αP), [19], the phosphorylation state of initiation factor eIF2α was assessed in NIL-2 and H19 cells that were lysed and analyzed by isoelectric focusing combined with immunoblotting as in [14]. The protein bands corresponding to eIF2α and eIF2αP were identified by a monoclonal antibody recognizing both forms of eIF2α (made by the late Dr. Henshaw, Rochester, USA and obtained via Dr. Proud, Dundee, UK). The phosphorylated form of eIF2α was not detected in NIL-2 or H19 cells, ruling out this possibility (J. Jiřička, unpublished).

4. Discussion

The studies described here reveal two important differences between v-*src*-transformed and non-transformed cells with respect to the regulation of proteins that modulate protein synthesis and the signalling events that impinge upon them. Firstly, transformed cells show a high basal activity of PI-3K. This is reflected in high basal activity of Akt, decreased activity of its downstream effector GSK-3 and increased activity of the regulatory translation factor eIF2B, which is phosphorylated and inhibited by GSK-3. Secondly, the high basal activity of p70 S6k in v-*src*-transformed cells appears to be independent of PI-3K, as indicated by its insensitivity to treatment of cells with wortmannin.

The high level of phosphorylation of 4E-BP1, a second target of mTOR signalling, also is not affected by wortmannin in v-*src*-transformed cells. Nevertheless, the fact that wortmannin treatment had no effect on the basal phosphorylation of 4E-BP1 in the non-transformed NIL-2 cells suggests that PI-3K is not involved in the control of 4E-BP1 phosphorylation in hamster fibroblasts.

The high activity of the PI-3K pathway may contribute to the dysregulation of cell growth in RSV-transformed cells. The present data show that eIF2B activity is enhanced in v-*src*-transformed cells, presumably due to increased activity of PI-3K and Akt, and consequent inactivation of GSK-3, a negative regulator of eIF2B [15,20]. eIF2B is a key regulator of overall protein synthesis and the increased activity of this factor in RSV-transformed cells is expected to contribute to increased activity of its substrate eIF2 and to enhanced rates of protein synthesis. The PI-3K/Akt signalling pathway has been shown to block apoptosis and promote cell survival and proliferation [21]. GSK-3, which is probably a central element in this survival pathway [22], controls several biosynthetic pathways and the key proteins of these processes are its substrates. Indeed, it has recently been suggested [23] that regulation of eIF2B via PI-3K/Akt/GSK-3 may play an important role in cell survival. Thus, the activation of eIF2B in v-*src*-transformed cells is expected to exert an antiapoptotic effect.

Previous studies have suggested a model in which PI-3K and PKB/Akt signal to p70 S6k and 4E-BP1 via mTOR. One possibility is that mTOR is directly regulated by Akt, an idea which is supported by the evidence that mTOR is a direct substrate of Akt [24]. Furthermore, recent data show that TSC2, which can inhibit mTOR signalling, is inactivated by Akt [5], providing an additional input from PI-3K/Akt into regulation of mTOR and its downstream effectors such as p70 S6k and 4E-BP1. Formation of the regulatory complex of p85 subunit of PI-3K with p70 S6k and mTOR [25] also suggests a role of PI-3K in the activation of p70 S6k in lymphoma cells. However, other data show that in *Drosophila*, p70 S6k activation is independent of PI-3K [26], and Düfner et al. [27] have presented data arguing against a role for Akt in the physiological regulation of p70 S6k.

We have previously shown that phosphorylation and activation of p70 S6k in both non-transformed NIL-2 cells and RSV-transformed H19 cells was completely blocked by rapamycin [7]. The observation that the phosphorylation of p70 S6k is sensitive to wortmannin in non-transformed NIL-2 cells, demonstrated in this paper, indicates that p70 S6k is downstream of PI-3K in normal hamster fibroblasts. In contrast, in RSV-transformed fibroblasts, wortmannin failed to decrease the phosphorylation of p70 S6k. These data suggest that the activation of p70 S6k is not the only or indeed the major event involved in the upregulation of translation mediated by PI-3K in hamster fibroblasts transformed by the v-*src* oncogene, and that important distinctions exist between the signalling pathways that modulate protein synthesis in the non-transformed cells and v-*src*-transformed cells.

In summary, we have found that at least two signalling pathways that control mRNA translation are activated by RSV transformation of hamster fibroblasts. The first is the rapamycin-sensitive mTOR signalling pathway, which regulates the phosphorylation and the activity of ribosomal S6 protein kinase p70 S6k and the phosphorylation of translational repressor protein 4E-BP1 [7], which is independent of PI-3K in v-*src*-transformed cells. The second is a distinct antiapoptotic PI-3K/Akt/GSK-3 signalling pathway that increases the activity of the regulatory initiation factor eIF2B (this work).

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