Identification of Insulin-Induced Sites of Ribosomal Protein S6 Phosphorylation in *Drosophila melanogaster*†

Thomas Radimerski,‡ Thierry Mini,§ Ulrich Schneider,§ Richard E. H. Wettenhall,|| George Thomas,‡ and Paul Jenö*,§

Friedrich Miescher Institute, Basel, Switzerland, Biozentrum of the University of Basel, Basel, Switzerland, and The Russell Grimwade School of Biochemistry and Molecular Biology, University of Melbourne, Melbourne, Australia

Received November 30, 1999; Revised Manuscript Received February 14, 2000

ABSTRACT: Insulin treatment of *Drosophila melanogaster* Kc 167 cells induces the multiple phosphorylation of a *Drosophila* ribosomal protein, as judged by its decreased electrophoretic mobility on two-dimensional polyacrylamide gels. The extent to which insulin induces this response is potentiated by cycloheximide and blocked by pretreatment with rapamycin. Isolation and mass spectrometric analysis revealed that the multiply phosphorylated protein was the larger of two *Drosophila melanogaster* orthologues of mammalian 40S ribosomal protein S6, termed here *D*S6A. Proteolytic cleavage of *D*S6A derived from stimulated Kc 167 cells with the endoproteinase Lys-C released a number of peptides, one of which contained all the putative phosphorylation sites. Conversion of phosphoserines to dehydroalanines with $Ba(OH)_2$ showed that the sites of phosphorylation reside at the carboxy terminus of *D*S6A. The sites of phosphorylation were identified by Edman degradation after conversion of the phosphoserine residues to *S*-ethylcysteine as Ser_{233}, Ser_{235}, Ser_{239}, Ser_{242}, and Ser_{245}. Finally, phosphopeptide mapping of individual phosphoderivatives, isolated from two-dimensional polyacrylamide gels, indicated that *D*S6A phosphorylation, in analogy to mammalian S6 phosphorylation, appears to proceed in an ordered fashion. The importance of these observations in cell growth and development is discussed.

Mitogenic stimulation of quiescent cells induces the activation of a number of anabolic processes which contribute to the ability of the cell to progress through the cell cycle, leading to cell growth, DNA synthesis, and cell division (*1*). The activation of these processes is triggered at the cell surface through ligand-induced activation of either tyrosine kinase receptors or G protein-coupled seven-transmembrane receptors (*2*). In the context of the mitogenic response, the activation and maintenance of high rates of protein synthesis represents an obligatory anabolic response (*3*). This response is thought to be propagated intracellularly by serine/threonine kinases, which phosphorylate a number of specific translation components (*4*). One of these components is the 40S ribosomal protein S6 whose increased phosphorylation is mediated by $p70^{s6k}$ and $p85^{s6k}$. These two kinases are translated from a common mRNA transcript by usage of alternative translation initiation start sites (unpublished data). Their amino acid sequences are identical, except for a 23-amino acid extension at the amino terminus of $p85^{s6k}$ which targets the larger isoform to the nucleus (*5*). As recent studies have led to the identification of a second S6K gene, termed S6K2 (*6*−*8*), $p70^{s6k}$ and $p85^{s6k}$ have been collectively termed S6K1 (*6*). S6K2 is highly homologous to the smaller S6K1 isoform, except that it contains a distinct carboxy terminus, which may be involved in nuclear localization as well as differential intracellular targeting. S6K1 activation appears to require a complex interplay between a number of specific phosphorylation sites and distinct intramolecular domains. Given the high homology and conservation of key phosphorylation sites, it is reasoned that S6K2 is regulated in a manner similar to S6K1. Consistent with this hypothesis, both kinases appear to reside on a common signaling pathway (*6*−*8*), which is initiated at the cell surface by the activation of phosphatidylinositide-3′OH kinase, PI3K (*9*).

Increased S6K activation and S6 phosphorylation are paralleled by the selective translational up-regulation of a class of mRNAs containing an oligopyrimidine tract at their transcriptional start site, termed a 5′TOP (*10*). The genes representing this family of mRNAs are small in number, containing no more than 200 members, but can account for up to 20% of total cellular mRNA. 5′TOP mRNAs encode for a number of components of the translational apparatus, including ribosomal proteins and translation elongation factors. Recent studies have shown that the up-regulation of these mRNAs is largely suppressed by the antibiotic rapamycin, a bacterial macrolide (*10*). In parallel, rapamycin also abolishes S6K activation, and subsequent mitogen-induced S6 phosphorylation (*10*). More importantly, the suppressive effects of rapamycin on 5′TOP translation can be rescued by coexpression of recombinant S6K1 rapamycin-resistant

† This work was supported in part by grants from the Swiss Cancer League, European Economic Community, and Human Frontier Science Organization to G.T. and by Swiss National Research Foundation Grant 3100-049416.96/1 to P.J.

* To whom correspondence should be addressed at the Department of Biochemistry, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland. Email: Paul.Jenoe@unibas.ch, Phone: +41 61 267 21 57, Fax: +41 61 267 21 48.

‡ Friedrich Miescher Institute.

§ Biozentrum of the University of Basel.

|| University of Melbourne.

10.1021/bi9927484 CCC: $19.00
Published on Web 04/22/2000

mutants, providing a causal link between S6K1 activation and translational up-regulation of 5′TOP mRNAs (*11*). The effects of S6K activation on the translation of 5′TOP mRNAs are thought to be mediated through the increased phosphorylation of S6. S6 itself has been mapped by protein−protein and RNA−protein cross-linking studies to the mRNA−tRNA binding site of the 40S ribosome (*12*), suggesting that it might have a role in the regulation of mRNA translation. Following mitogenic stimulation, up to 5 mol of phosphate is incorporated in an ordered fashion into mammalian S6 (*13*). In mammals, the phosphorylation sites have been mapped to a short sequence at the carboxy terminus of the molecule (*14*), with phosphorylation progressing in an ordered fashion from $Ser_{236} \rightarrow Ser_{235} \rightarrow Ser_{240} \rightarrow Ser_{244}$ and Ser_{247} (*14, 15*).

Although S6 is hypothesized to be the downstream effector of S6K activation, corroborative in vivo data are lacking. The recent identification of an S6K homologue in *Drosophila melanogaster* (*16, 17*) has led to the hypothesis that the pathway controlling translation of ribosomal proteins is conserved among insects and mammals. Given the conservation of signaling pathways in *Drosophila*, combined with its powerful genetics and lower gene number, it was reasoned that *Drosophila* would prove useful in unraveling the role of S6 phosphorylation as an effector of S6K function. Indeed, S6 phosphorylation has been described in a number of insect systems, including (i) the silk worm *Bombyx mori*, following refeeding after fasting (*18*), (ii) the tobacco hornworm *Manduca sexta*, following induction by prothoracicotropic hormone (PTTH) (*19, 20*) and (iii) in cells from the fall army worm *Spodoptera frugiperda*, after baculovirus infection (*21*). In all three insect systems, there appears to be 5 mol of phosphate incorporated per mol of S6, as judged from its reduced electrophoretic mobility on two-dimensional polyacrylamide gels. However, in *Drosophila melanogaster*, the results are less clear. First, only one phosphoderivative of the putative *Drosophila* S6 homologue was reported (*22*). Second, the recently reported genomic sequence of the *D*S6[1] gene has revealed that there are two tandem alternative exon repeats downstream of exon 3A. If either of these repeats are utilized, as has been reported (*23*), they would give rise to a distinct protein product of significantly shorter length and protein sequence (*24, 25*). Finally, a P-element insertion in the 5′UTR of the *D*S6 gene, which is X chromosome-linked, leads to a decrease in *D*S6 gene expression, with hemizygous males and homozygous females failing to emerge as adults after a prolonged third larval instar (*24, 25*). During larval development, the *D*S6 mutants develop melanotic tumors in the hematopoietic system with significantly overgrown lymph glands (*25*), implying an additional role for *D*S6 in tumor suppression of larval hemocytes, which mediate the immune response in insects (*25*).

As a basis to exploit *Drosophila melanogaster* in future investigations on the role of S6 phosphorylation in cell growth and development, we first set out to determine whether the protein is phosphorylated in response to insulin treatment and to identify which form of *D*S6 was present in *Drosophila* 40S ribosomal subunits. Next, following digestion of the protein with endoproteinase LysC, a combination of electrospray ionization mass spectrometry and chemical sequencing was employed to identify the phosphorylated peptides and the sites of phosphorylation. Finally, phosphopeptide analysis of the increasingly phosphorylated *D*S6 derivatives isolated from two-dimensional polyacrylamide gels was utilized to assess whether in analogy to vertebrate systems, *D*S6 phosphorylation proceeded in an ordered fashion.

EXPERIMENTAL PROCEDURES

Cell Culture and in Vivo [^{32}P]P_i Labeling. *Drosophila* Kc 167 cells were maintained at 25 °C in *Drosophila* Schneiders medium containing 15% heat-inactivated fetal bovine serum, 1% (v/v) antibiotic antimycotic, 1% (v/v) gentamicin, 4 mM glutamine, 7.4 μM penicillin, and 1.4 μM streptomycin. Cells were seeded at 5×10^6 cells/mL in a volume of 25 mL and were subcultured every third or fourth day. Stationary phase cells were stimulated with 100 nM insulin either alone or in combination with 100 μM cycloheximide for 2 h. For [^{32}P]-P_i labeling, cells were first suspended in 15 mL of phosphate-free Dulbecco's modified Eagle's medium (DMEM) containing 15% dialyzed and heat-inactivated fetal bovine serum and 4 mM glutamine. The cells were incubated for 4 h with 2 mCi of [^{32}P]P_i (10 mCi/mL $^{32}PO_4$) per dish and then stimulated for a further 2 h with a combination of 100 nM insulin and 100 μM cycloheximide. Alternatively, in some cases, 2 h prior to stimulation, the cells were pretreated with 1 μM okadaic acid. Following [^{32}P]P_i labeling, cells were washed with ice-cold PBS and lysed, and extraction of ribosomes was carried out as described below.

Isolation of Ribosomes and DS6K Assays. For preparation of ribosomes, cells were harvested from four dishes by centrifugation (2500 rpm for 5 min), resuspended in 6 mL of hypotonic buffer (5 mM Tris-HCl, pH 7.4, 1.5 mM KCl, 2.5 mM $MgCl_2$), and lysed with a polytron homogenizer (Kinematica, Switzerland) for 10 s (setting 5). The homogenate was diluted to 12 mL with lysis buffer containing 2% sodium deoxycholate (DOC), 1% Triton X-100, and 40 mM *p*-nitrophenyl phosphate (pNPP) and homogenized a second time. Cell debris was removed by spinning the homogenate twice at 10 000 rpm for 15 min in a Sorvall SS34 rotor at 4 °C. The resulting supernatant was split in two and transferred separately into Beckman Quick Seal centrifugation tubes. The homogenate was successively underlaid with 2 mL of 0.5 M sucrose solution and 2 mL of 1.1 M sucrose solution. The sucrose solutions were prepared in a buffer containing 0.5 mM Tris-HCl, pH 7.4, 0.25 mM $MgCl_2$, 0.5 M KCl, 1% Na-DOC, 1% Triton X-100, and 20 mM pNPP. Total ribosomes were harvested by centrifugation in a Beckman 70.1 Ti rotor at 62 500 rpm for 16 h at 4 °C. The pellet was washed once with 200 μL of Staehelin A buffer (20 mM Tris-HCl, pH 7.4, 100 mM KCl, 5 mM $MgCl_2$, 1 mM DTT). The ribosomes were resuspended in 700 μL of Staehelin A buffer by shaking the tube at 4 °C for 3−4 h on an orbital shaker (Mini-Shaker, Adolf Kühner AG, Basel, Switzerland) set at 350 rpm. Several 4 mm glass

[1] Abbreviations: *D*S6, *Drosophila melanogaster* ribosomal protein S6; *D*S6K, *Drosophila melanogaster* ribosomal protein S6 kinase; ESI/MS, electrospray ionization mass spectrometry; LC/MS, liquid chromatography coupled to mass spectrometry; LC/MS/MS, liquid chromatography coupled to tandem mass spectrometry; *m*/*z*, mass-to-charge ratio; RP-HPLC, reverse-phase high-pressure liquid chromatography; TFA, trifluoroacetic acid; TLE, thin-layer electrophoresis; 2D-TLE, two-dimensional thin-layer electrophoresis.

beads were added to the tube to facilitate complete resuspension. *D*S6K activity from *Drosophila* Kc 167 cell extracts was measured as described (*17*).

Two-Dimensional Polyacrylamide Gel Electrophoresis of Ribosomal Proteins. Total ribosomal proteins from *Drosophila* Kc 167 cells were isolated and separated by two-dimensional polyacrylamide urea gel electrophoresis employing the standard protocols (*26*), as internationally agreed for their identification (*27*), except that for the second dimension, proteins were first electrophoresed for 2 h at 50 V and then 22 h at 200 V. For protein visualization, the gels were stained in 0.1% Coomassie Blue R250, 50% methanol, 10% acetic acid for 45 min at 45 °C, followed by destaining in 20% methanol, 10% acetic acid.

Reverse-Phase High-Performance Liquid Chromatography of Ribosomal Proteins. Ribosomal proteins were extracted by mixing a 10 μL aliquot of total ribosomes (20 μg/μL) with 10 μL of 6 M guanidinium chloride (GdmCl) followed by the addition of 80 μL of 0.2% TFA (*28*). After centrifugation at 13 000 rpm for 5 min, the supernatant was injected onto a Vydac C_{18} 218TP52 (2.1 × 250 mm) column which had been equilibrated in 0.1% TFA. Bound proteins were eluted at 150 μL/min with a linear gradient up to 60% acetonitrile, containing 0.09% TFA during 60 min. Proteins were detected at 214 nm and were collected manually for further analysis. The elution of ribosomal protein *D*S6 was followed by mixing ribosomes from stimulated or from rapamycin-treated cells with ribosomes isolated from cells which had been labeled in vivo with [^{32}P]P_i. Radioactivity was measured by Cerenkov or liquid scintillation counting. Preparative isolation of the carboxy-terminal peptide comprising residues 230−248 from endoproteinase LysC digests of *D*S6A was carried out on a Vydac C_{18} 218TP51 (1.0 × 250 mm) column at a flow rate of 50 μL/min with linear gradients from 0 to 60% acetonitrile in 45 min in either 0.1% TFA or 10 mM NaH_2PO_4/Na_2HPO_4, pH 6.5. Capillary reverse-phase chromatography of enzymatic digests of *D*S6A coupled to ESI/MS was carried out on 100 μm i.d. columns packed into fused silica capillaries with either C_{18} 218TP material (5 μm particle size, Vydac, The Separations Group, Hesperia, CA) or C_{18} Monitor-Spherical Silica (5 μm particle size, Column Engineering Inc., Ontario, CA) (*29*). The capillary columns were developed with 0.05% TFA to 60% acetonitrile containing 0.05% TFA at a flow rate of 1−2 μL/min. The effluent was directed into a microspray ionization source (*30*).

Enzymatic Digestions. Ribosomal protein *D*S6 isolated by RP-HPLC was dried in a speedvac concentrator, redissolved in 20 μL of 100 mM Tris-HCl, pH 8.0, 6 M urea, and digested with 500 ng of endoproteinase LysC (*Achromobacter* Protease I, Wako Chemicals) for 1 h at 37 °C. The reaction was stopped by adding TFA from a 4% stock solution to a final concentration of 0.2%. For phosphopeptide analysis, S6 was digested with 1 μg of endoproteinase LysC in 20 μL of 20 mM $(NH_4)HCO_3$ for 2 h at 37 °C. The reaction was stopped by the addition of 200 μL of water and immediately frozen in liquid nitrogen. The sample was dried in a speedvac concentrator, and the pellet was washed with water, dried again, and resuspended in 10 μL of 130 mM $(NH_4)HCO_3$, pH 8.9. In-gel digestion of S6 phosphoderivatives separated by two-dimensional polyacrylamide gel electrophoresis was carried out by cutting out the individual spots with a razor blade. The gel pieces were washed twice with 40% 1-propanol, resuspended in 250 μL of 20 mM $(NH_4)HCO_3$ containing 5 μg of endoproteinase LysC, and digested for 12 h at 37 °C. The supernatant was dried, and the peptides were dissolved in 10 μL of 130 mM $(NH_4)HCO_3$, pH 8.9.

Two-Dimensional Phosphopeptide Analysis. Radioactively labeled phosphopeptides were separated on TLC plates (Merck) in a HTLE 7000 flatbed apparatus (CBS Scientific, Inc.). First-dimension electrophoresis was at pH 8.9 in 126 mM $(NH_4)HCO_3$ for 30 min at 1000 V, using xylene cyanole as the tracking dye. The second-dimension electrophoresis was at pH 1.9 in a buffer consisting of 2.5% formic acid, 7.8% acetic acid for 30 min at 1000 V using DNP-lysine as the tracking dye (*31*). After drying the plates, phosphopeptides were visualized using a PhosphorImager and ImageQuant Software (Molecular Dynamics).

Chemical Dephosphorylation and Edman Degradation. Conversion of P-Ser to dehydroalanine was carried out by adding a small crystal of $Ba(OH)_2$ to endoproteinase LysC digests of phosphorylated S6 and incubation of the digest at 30 °C for 90 min (*32*). The reaction was stopped by adding 10% acetic acid to a final concentration of 1%. Alternatively, P-Ser-containing S6 peptides were converted to *S*-ethylcysteine peptides essentially as described (*33*). Peptides were sequenced by automated Edman degradation using a model 430A protein sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA). Solid-phase sequencing of [^{32}P]P_i-labeled phosphopeptides was carried out as described (*35*).

Mass Spectral Analysis. The microspray needles used for electrospray ionization were pulled from 100 μm i.d. × 280 μm o.d. fused silica capillaries (LC Packings, Amsterdam, The Netherlands) on a Model P-2000 quartz micropipette puller (Sutter Instrument Co., Novato, CA). For LC/MS analysis, the needles were placed into an XYZ micropositioner, and the voltage was applied directly to the sample stream through the capillary union (*30*). Spray voltages were usually between 1100 and 1400 V. Mass determinations were carried out on a TSQ7000 triple quadrupole mass spectrometer (Finnigan, San José, CA). All measurements were carried out in the positive ion mode. Precursor ion scanning was between 200 and 2000 Da for 3 s at unit resolution. For operation in the MS/MS mode, the resolution of Q1 was set to transmit a mass window of 4 Da and the resolution of Q3 was adjusted to 1.5 Da. Scanning was between 50 and 2250 Da in 3.5 s. Argon was used as a collision gas at a pressure of 3.5 mTorr.

Peptide and Ion Nomenclature. Peptides generated by endoproteinase LysC are labeled with K. The peptides are numbered according to their position based on the *Drosophila melanogaster* sequence (*34*). When ionized peptides are observed by MS, they are referred to as peptide or parent ions and their masses are represented by $[M + nH]^{n+}$ where *n* represents the number of protons that have been added to the peptide to achieve a charge of *n*. Treatment of $Ba(OH)_2$ to β-eliminate H_3PO_4 from phosphorylated serine yields a peptide mass that is 18 Da less than serine or threonine (β-elimination is equivalent to the loss of a molecule of water). β-Eliminated peptides are indicated by the symbol Δ followed by the number of how many phosphates were eliminated.

Drosophila melanogaster	RRRSASIRESKSSVSSDKK[248]
Drosophila alternative isoform	RGRYVTIRKPKSSVFSGKK[189]
Manduca sexta	RRRSASMRDSKSSNQSAPQK[253]
Homo sapiens	RRRLSSLRASTSKSESSQK[249]
Xenopus laevis	RRRLSSLRASTSKSESSQK[249]
Gallus gallus	RRRLSSLRASTSKSESSQK[249]
Rattus norvegicus	RRRLSSLRASTSKSESSQK[249]
Mus musculus	RRRLSSLRASTSKSESSQK[249]
Schizosaccharomyces pombe	ARRASSLKK[239]
Saccharomyces cerevisiae	KRRASSLKA[236]

FIGURE 1: Sequence comparison of the carboxy-terminal domains of ribosomal protein S6 from insects, vertebrates, and yeast. Amino acids in boldface type and underlined represent identified phosphorylation sites; amino acids in boldface type represent presumed phosphorylation sites based on sequence homology.

RESULTS

S6 Phosphorylation in Drosophila Kc 167 Cells. In all metazoan systems studied to date, mitogen-induced cell proliferation leads to multiple phosphorylation of 40S ribosomal protein S6 (*36*). In addition, extensive S6 phosphorylation has been reported in a number of insects (*18*−*21*), although in *Drosophila* Kc cells only a single phosphoderivative was observed (*22*). Sequence alignment of ribosomal protein S6 from *Drosophila* and vertebrates shows that all vertebrate S6 proteins studied to date have identical sequences surrounding their carboxy-terminal phosphorylation sites, whereas the carboxy-terminal domain in the two potential *Drosophila* orthologues is distinct (Figure 1). To determine whether mitogenic stimulation of Kc 167 cells induces the phosphorylation of either of these two proteins, termed *D*S6A and *D*S6B, total ribosomes isolated from exponentially growing cells were separated by two-dimensional polyacrylamide gel electrophoresis. The protein pattern was compared to the pattern obtained from ribosomes isolated from insulin, from insulin/cycloheximide, or from rapamycin-treated cells. From exponentially growing cells, one protein was separated into six distinct derivatives (Figure 2A), characteristic of multiple phosphorylation events associated with mammalian S6 phosphorylation (*14*). Increased levels of the slower migrating derivatives could be achieved by treating Kc 167 cells with 100 nM insulin alone or in combination with 100 μM cycloheximide (Figure 2B and 2C, respectively). Cycloheximide is known to induce up to 5 mol of phosphate per mole of ribosomal protein S6 in rat liver (*14*), and in subsequent studies carried out here, it was employed with insulin to ensure high levels of phosphorylation. Also, consistent with findings in vertebrate systems, treatment of exponentially growing Kc 167 cells with the immunosuppressant rapamycin, an agent known to block both S6 phosphorylation and S6K1/S6K2 activation in mammalian systems (*37*), led to the disappearance of the presumed phosphoderivatives (Figure 2D). A similar response was also observed when treating the Kc 167 cells with rapamycin 15 min prior to stimulation with insulin (data not shown). In vivo [^{32}P]P_i labeling of Kc 167 cells treated with insulin and cycloheximide resulted in the labeling of a single ribosomal protein whose migration coincided exactly with the presumed phosphoderivative *e* seen by Coomassie

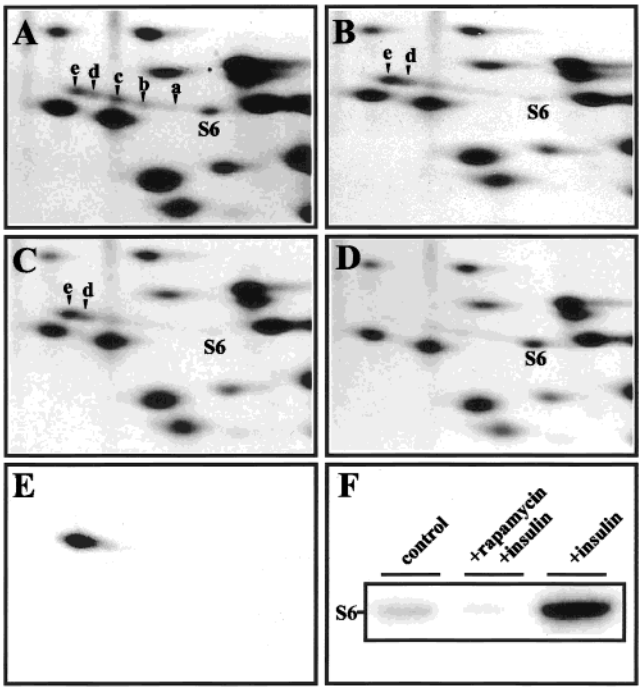

FIGURE 2: Two-dimensional polyacrylamide gel electrophoresis of *Drosophila* ribosomal proteins. (A) Ribosomal proteins from exponentially growing Kc 167 cells. S6 marks the position of unphosphorylated *D*S6; the spots labeled with *a*, *b*, *c*, *d*, and *e* mark the position of *D*S6 carrying 1, 2, 3, 4, and 5 phosphates. (B) Ribosomal proteins from Kc 167 cells treated with 100 nM insulin for 90 min. (C) Ribosomal proteins from Kc 167 cells which had been stimulated with 100 nM insulin/100 μM cycloheximide for 2 h. (D) Ribosomal proteins from exponentially growing Kc 167 cells which had been treated with 20 nM rapamycin. (E) PhosphorImage of two-dimensional gel electrophoresis separated ribosomal proteins from Kc 167 cells which had been labeled with [^{32}P]P_i in vivo prior to stimulation with 100 nM insulin/100 μM cycloheximide. To better visualize the influence of phosphorylation on the migration behavior of ribosomal protein *D*S6, only the portion of the gel in the vicinity of *D*S6 is shown. (F) *D*S6K activity assay. Control: untreated stationary Kc 167 cells. Rapamycin/insulin: Kc 167 cells pretreated with 20 nM rapamycin 15 min prior to stimulation with 1 μM insulin for 30 min. Insulin: *D*S6K activity in Kc 167 cells stimulated with 1 μM insulin for 30 min.

Blue staining (Figure 2E). Consistent with these findings, insulin stimulation induced *D*S6K activation in Kc 167 cells (Figure 2F) which could be completely blocked by pretreating the cells with rapamycin prior to stimulation with insulin (Figure 2F). Thus, together these results suggest that 5 mol of phosphate is incorporated into an apparent *Drosophila* S6 orthologue in response to mitogens.

Identification of DS6 by LC/MS/MS. To determine if the highly phosphorylated protein was equivalent to either *D*S6A or *D*S6B, a portion of the [^{32}P]P_i-labeled ribosomes from insulin- and cycloheximide-stimulated cells (Figure 2E) was mixed with total ribosomes isolated from rapamycin-treated Kc 167 cells, and the proteins were separated by RP-HPLC (*28*). Ribosomes from rapamycin-treated cells were employed to avoid ambiguities in the identification of the presumed carboxy-terminal peptides of either *D*S6A or *D*S6B caused by the presence of phosphorylated residues. Analysis of the fractionated proteins from the reverse-phase column revealed a single radioactive peak eluting at approximately 40% acetonitrile (Figure 3). Examination of the radioactive peak fractions by SDS−PAGE (Figure 3A) showed a unique radioactive protein which overlapped with a Coomassie-

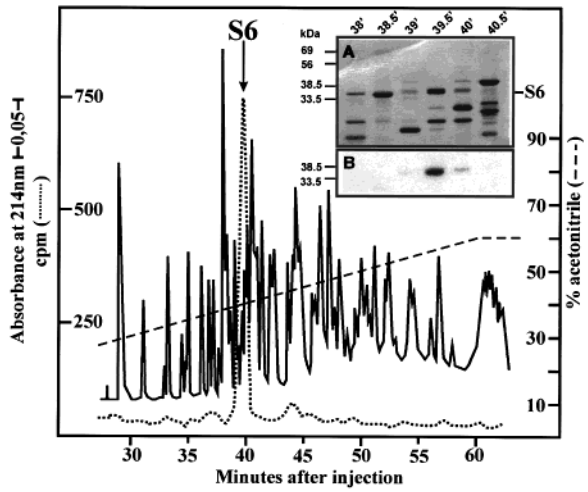

FIGURE 3: Reverse-phase HPLC separation of 80S ribosomal proteins from Kc 167 cells on a C_{18} column which had been labeled with [^{32}P]P_i prior to stimulation with insulin/cycloheximide. The dotted line shows the elution of radioactivity while the stippled line indicates the gradient applied to the column to elute the proteins. Inset A: SDS−PAGE analysis of fractions collected to locate the elution position of *D*S6. The retention times of the fractions are indicated above the inset. The fraction eluting after 39.5 min was found to contain *D*S6 as evidenced by the presence of a major band with an apparent molecular mass of 35 kDa upon Coomassie Blue staining. Inset B: PhosphorImage of the stained gel showing a single radioactively labeled protein with an apparent molecular mass of 35 kDa.

stained protein of approximately 30 kDa (Figure 3B), a molecular mass similar to that of the larger *D*S6A splice product (*34*). At least three other ribosomal proteins coeluted with the presumed *D*S6 whose identities were not determined. When the fraction containing the radioactivity was digested with the endoproteinase LysC and analyzed by automated LC/MS/MS, 18 peptides could be unambiguously identified as being derived from the larger *D*S6A isoform (Table 1). Interestingly, two peptides, K_{35} and incompletely cleaved K_{36-37}, encompassing the potential phosphorylation sites were clearly identified during the LC/MS/MS run (Table 1, Figure 4A). The latter peptide arises from incomplete cleavage at Lys 247 (Figure 4A). Taken together, the findings are consistent with *D*S6A being associated with *Drosophila* ribosomes and becoming multiply phosphorylated in response to insulin treatment.

DS6A Phosphorylation Probed by ESI/MS. Previously, the sites of phosphorylation in rat ribosomal protein S6 have been shown to reside at the carboxy terminus preceded by a highly basic sequence which serves as a recognition motif for S6K1 (*15*). Sequence alignment of the rat and the *Drosophila* protein reveals that the same basic recognition motif exists in *D*S6A, followed by a stretch of amino acids containing seven serines (Figure 1). To determine whether these residues serve as phosphorylation sites in *D*S6A, larger amounts of the protein were isolated from Kc 167 cells stimulated with insulin and cycloheximide, digested for 1 h with the endoproteinase LysC, and subjected to LC/MS analysis. Depending on the extent of cleavage, a number of peptides covering the potential sites of phosphorylation were expected, including residues 230−240 (K_{35}), 241−247 (K_{36}), 241−248 (K_{36-37}), 230−247 (K_{35-36}), and 230−248 (K_{35-37}) (Figure 4A). After separation of the digest by capillary chromatography coupled to a micro electrospray ionization source (*30*), the ion tracing was searched for the presence of these peptides. Weak signals for unphosphorylated and singly phosphorylated K_{35} were detected, possibly originating from residual unphosphorylated *D*S6A. In addition, weak signals for incompletely cleaved K_{35-37} peptide containing one, two, and three phosphates were also detected. While the ion intensities for the singly, doubly, and triply phosphorylated K_{35-37} peptide decreased with increasing number of phosphates, two-dimensional gel electrophoretic analysis of ribosomes from insulin- and cycloheximide-treated cells indicated a substantially higher degree of phosphorylation than detected by LC/MS analysis. The most likely explanation for this finding is that ionization of the highly phosphorylated peptides was selectively suppressed during the electrospray process. Such problems can be circumvented by conversion of phosphoserine to dehydroalanine by β-elimination of H_3PO_4 with $Ba(OH)_2$, allowing detection of phosphorylated peptides by electrospray ionization (*38*). Therefore, an endoproteinase LysC digest of *D*S6A from insulin- and cycloheximide-stimulated Kc 167 cells was treated with $Ba(OH)_2$ and subsequently separated by capillary LC/MS. Searching the ion tracing for all possible combina-

Table 1: Calculated and Measured Masses of the Endoproteinase LysC Peptides Obtained from *Drosophila* S6 from Rapamycin-Treated Cells[a]

peptide	residues	scan no.	calculated mass (Da)	measured mass (Da)
K_1	1−2	n.o.	278.2	n.o.
K_2	3−14	307	1280.6	2981.1[b]
K_3	15−23	294	1115.6	1115.0
K_4	24−30	296	954.5	954.4
K_5	31−46	319	1845.9	1845.9
K_6	47−58	282	1291.7	1291.4
K_7	59−64	270	707.4	706.5
K_8	65−78	315	1590.0	1589.9
K_9	79−79	n.o.	147.1	n.o.
K_{10}	80−93	307	1702.9	2981.1[b]
K_{11}	94−95	n.o.	303.2	n.o.
K_{12}	96−115	n.o.	2087.2	n.o.
K_{13}	116−116	n.o.	147.1	n.o.
K_{14}	117−119	n.o.	333.2	n.o.
K_{15}	120−136	304	1850.1	1850.0
K_{16}	137−140	n.o.	461.3	n.o.
K_{17}	141−143	n.o.	416.3	n.o.
K_{18}	144−149	268	737.4	736.5
K_{19}	150−165	304	1953.1	1952.8
K_{20}	166−168	n.o.	376.2	n.o.
K_{21}	169−169	n.o.	147.1	n.o.
K_{22}	170−173	n.o.	406.2	n.o.
K_{23}	174−176	n.o.	315.2	n.o.
K_{24}	177−189	308	1564.0	1564.1
K_{25}	190−196	295	893.6	892.5
K_{26}	197−197	n.o.	147.1	n.o.
K_{27}	198−198	n.o.	147.1	n.o.
K_{28}	199−204	249	702.4	701.2
K_{29}	205−212	233	854.4	853.4
K_{30}	213−218	277	765.5	755.6
K_{31}	219−219	n.o.	147.1	n.o.
K_{32}	220−222	n.o.	363.2	n.o.
K_{33}	223−224	n.o.	218.2	n.o.
K_{34}	225−229	n.o.	632.3	n.o.
K_{35}	230−240	290	1345.8	1345.1
K_{36}	241−247	238	709.3	836.5[c]
K_{37}	248−248	n.o.	147.1	n.o.

[a] The elution position of peptides is denoted by the corresponding scan number (each scan is 3 s). n.o. indicates fragments which were not observed during the LC/MS/MS analysis. [b] K_2 was found to be disulfide-bridged with K_{10}. [c] The measured mass corresponds to K_{36-37}.

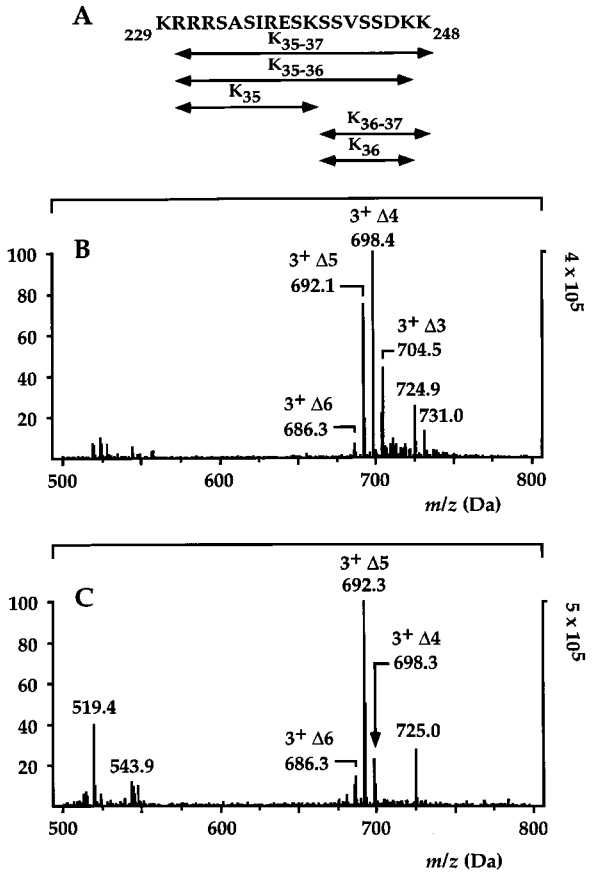

FIGURE 4: (A) Carboxy-terminal sequence of *D*S6A comprising residues 229−248 and expected peptides generated upon complete (K_{35} and K_{36}) and incomplete (K_{35-36}, K_{35-37}, K_{36-37}) endoproteinase LysC cleavage. (B) Mass spectrum of $Ba(OH)_2$-treated RRRSASIRESKSSVSSDKK (K_{35-37}) from cells stimulated with insulin and cycloheximide. The spectrum was obtained by summing up all scans acquired during the elution of Δ3−Δ5 K_{35-37}. For better clarity, only the portion of the spectrum corresponding to the triply charged peptides is shown. Δ6, Δ5, Δ4, and Δ3 indicate the number of dehydroalanines arising from $Ba(OH)_2$ treatment of K_{35-37} carrying 6, 5, 4, and 3 phosphates. (C) Spectrum of $Ba(OH)_2$-treated K_{35-37} from cells which had been treated with okadaic acid prior to insulin/cycloheximide treatment to suppress phosphatase activity. For labeling of the spectrum, see panel B.

tions of K_{35} and incompletely cleaved K_{36-37} and K_{35-37} revealed strong signals with mass-to-charge ratios of 692.1, 698.4, and 704.5 Da, corresponding to the triply charged ions of the RRRSASIRESKSSVSSDKK (K_{35-37}) peptide containing three, four, and five dehydroalanines. The intensity of the corresponding ions closely resembles the staining intensity of the individual phosphoderivatives of *D*S6A, with the tetra-dehydroalanine derivative being the most abundant species and equivalent to the quadruply phosphorylated *D*S6A (compare Figures 4B and 2C). Furthermore, when *D*S6A was isolated from cells which had been pretreated with okadaic acid prior to stimulation (Experimental Procedures), substantially higher amounts of the penta-dehydroalanine derivative could be observed in the spectrum of the carboxy-terminal K_{35-37} peptide (Figure 4C). Indeed, weak signals for a hexa-dehydroalanine derivative also increased under these conditions (Figure 4B,C), which may indicate a previously undetected low stoichiometry phosphorylation site in the same peptide. The results show that, in analogy to rat S6 (*14*), the phosphorylation sites in the *Drosophila* protein are located at the carboxy terminus, between residues 230

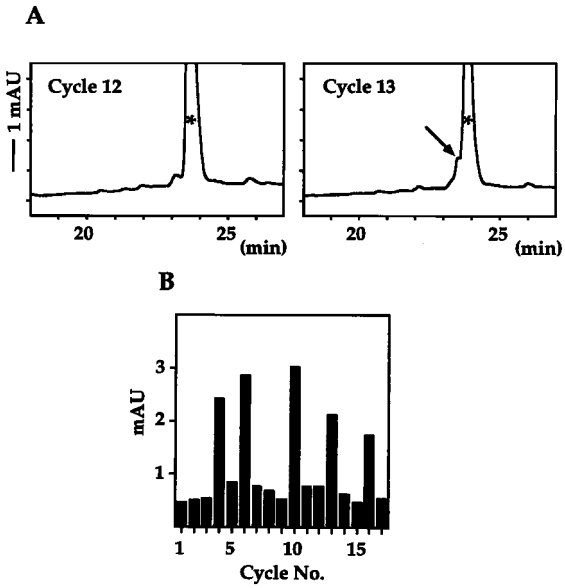

FIGURE 5: Identification of the *S*-ethylcysteine residues in RRRSASIRESKSSVSSDKK (K_{35-37}) peptide of *D*S6A (residues 230−248). Phosphoserine residues were converted to *S*-ethylcysteine as described under Experimental Procedures. (A) PTH-amino acid analysis of two rounds of Edman degradation: cycle 12 corresponding to Ser_{241} showing no release of PTH-*S*-ethylcysteine and cycle 13 (corresponding to Ser_{242}) showing the presence of PTH-*S*-ethylcysteine (labeled with an arrow) which elutes close to diphenylthiourea (DPTU, labeled with an asterisk), a major breakdown product of the Edman chemistry. (B) Graphic representation of the peak heights (background-corrected) of all PTH-*S*-ethylcysteines detected during K_{35-37} sequencing. The PTH-*S*-ethylcysteines correspond to the following residues: cycle 4, Ser_{233}; cycle 6, Ser_{235}; cycle 10, Ser_{239}; cycle 13, Ser_{242}; and cycle 16, Ser_{245}.

and 248, and in agreement with the two-dimensional polyacrylamide gel electrophoresis (Figure 2), that up to five phosphates, and possibly a sixth, are incorporated into *D*S6A.

Localization of the Sites of Phosphorylation of DS6A. The sequence stretch between residues 230 and 248 contains seven serines. Unfortunately, due to the very basic nature of the K_{35-37} peptide, substantial fragmentation in the collision cell of the mass spectrometer could not be induced even at the highest collision energy (−50 eV), precluding this method for identifying the sites of *D*S6A phosphorylation. Therefore, a combination of chemical- and radiosequencing methods was utilized. For this, Kc 167 cells were prelabeled with [^{32}P]P_i, and total ribosomal proteins were isolated following insulin and cycloheximide stimulation. Radiolabeled *D*S6A was partially purified by reverse-phase HPLC and cleaved with endoproteinase LysC, and the digest was separated on a C_{18} column at pH 6.5. Due to its high polarity, the phosphorylated K_{35-37} peptide was the first peptide to elute from the column. To obtain residues which can easily be assigned to phosphorylation sites during the chemical sequencing procedure, the phosphoserines of the K_{35-37} peptide were converted to *S*-ethylcysteine residues (*33*) and subjected to N-terminal sequencing. In this way, a peptide sequence corresponding to *D*S6A residues 230−248, RRRSASIRESKSSVSSDKK, could clearly be deduced from the corresponding degradation cycles. PTH-*S*-ethylcysteine was detected at cycles 4, 6, 10, 13, and 16 (Figure 5), showing that the sites of phosphorylation are Ser_{233}, Ser_{235}, Ser_{239}, Ser_{242}, and Ser_{245}. These results were corroborated

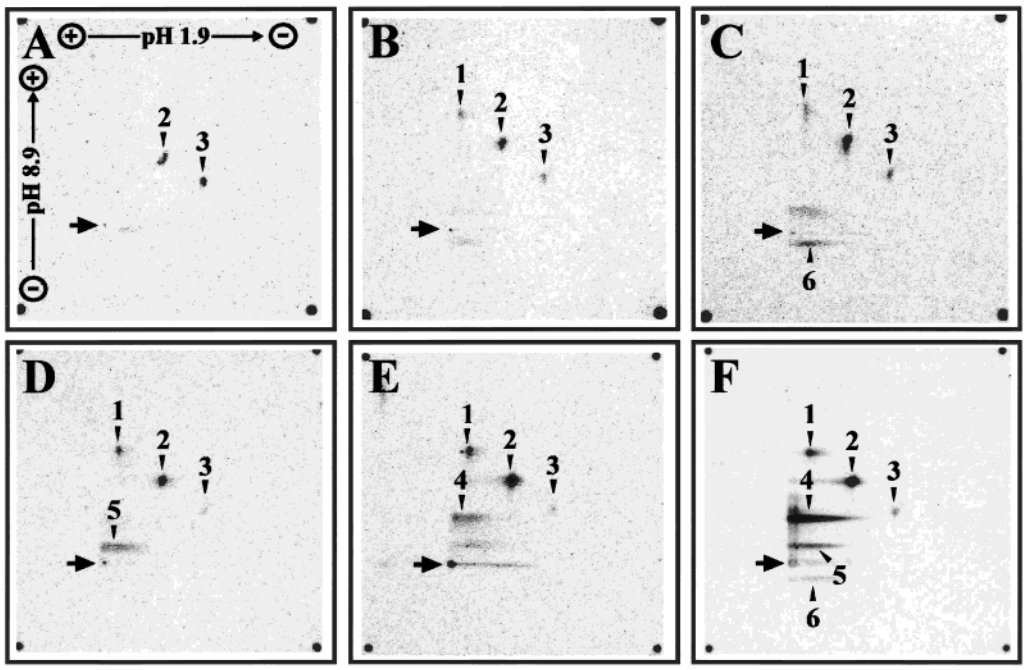

FIGURE 6: Phosphopeptide mapping of *D*S6A. (A−E) The [^{32}P]P_i-labeled phosphoderivatives *a*−*e* which had been cut individually from the two-dimensional gel (see Figure 2) were digested with the endoproteinase LysC and analyzed individually by 2D-TLE. (F) *D*S6A from [^{32}P]P_i-labeled Kc 167 cells was isolated by RP-HPLC, digested with endoproteinase LysC, and subjected to 2D-TLE. The arrow indicates the sample origin. The first dimension was from bottom to top; the second dimension was from left to right as described under Experimental Procedures.

by N-terminal solid-phase sequencing (*35*), showing release of radioactive phosphate at cycles 4, 6, 10, 13, and 16 (results not shown). However, in neither case could a sixth phosphorylation site be detected, possibly due to low stoichiometry. Thus, as in the case of mammalian S6, five specific sites of phosphorylation could be detected.

Phosphorylation of DS6A Proceeds in an Ordered Fashion. In mammalian systems, S6 phosphorylation by S6K1 has been shown to be an ordered process (*13*). To determine if phosphorylation of *D*S6A may occur in a similar fashion, Kc 167 cells were labeled in vivo with [^{32}P]P_i followed by stimulation with insulin and cycloheximide. Total ribosomal proteins were prepared and subjected to two-dimensional polyacrylamide electrophoresis, and the radioactively labeled phosphoderivatives *a*−*e* were excised individually (see Figure 2A) and digested extensively overnight (Experimental Procedures) with endoproteinase LysC and the resulting peptides separated by two-dimensional TLE (*13*). Inspection of the phosphopeptide patterns obtained from the individual phosphoderivatives of *D*S6A showed that a unique set of peptides is generated for each individual phosphoderivative (Figure 6 A−E). Analysis of phosphoderivative *a* revealed two phosphopeptides, 2 and 3 (Figure 6A), which may represent overlapping peptides containing the same site of phosphorylation generated by extensive digestion of *D*S6A. Similar results were previously observed for mammalian S6 (*13*). Digestion of phosphoderivative *b* released, in addition to the previously observed spots, phosphopeptide 1 (Figure 6B) and possibly a trace amount of phosphopeptide 6. The appearance of phosphopeptide 6 is probably explained by the overlapping of the increasingly phosphorylated derivatives on the two-dimensional polyacrylamide gels (see Figure 2A). Analysis of phosphoderivatives *c*, *d*, and *e* revealed phosphopeptides 6, 5, and 4, respectively (Figure 6C−E). Furthermore, when fully phosphorylated *D*S6A from insulin- and cycloheximide-stimulated cells was isolated by RP-HPLC, a set of phosphopeptides resembling that of Figure 6E was generated with endoproteinase LysC (Figure 6F). Although the identity of the individual phosphopeptides has not been established, it appears that, in analogy to mammalian S6, phosphorylation of *D*S6A proceeds in an ordered fashion (see Discussion).

DISCUSSION

Here we have used a combination of two-dimensional gel electrophoresis ESI/MS and solid-phase sequencing to identify the S6 phosphorylation sites after insulin stimulation of Kc 167 cells. The search for phosphorylation sites in proteins by mass spectrometry can be carried out by measuring the masses of all peptides from a digest and searching for those peptides whose mass is increased by 80 Da, the mass of a phosphate group (*38*, *40*). The loss of phosphoric acid upon fragmentation is often taken as proof that the peptide is phosphorylated (*41*). Here, the level of *D*S6A phosphorylation was difficult to assess by electrospray ionization; the reason for this is that cleavage of the carboxy terminus of *D*S6A by endoproteinase LysC is apparently dependent on the extent of *D*S6A phosphorylation producing a complex mixture of partially cleaved forms with varying extents of phosphorylation (Figure 4A). This problem was partially circumvented by analyzing the highly phosphorylated K_{35-37} peptide; however, higher phosphorylated *D*S6A peptides yielded progressively weaker ion intensities (results not shown). Although poor chromatographic recovery of phosphopeptides has been reported due to selective binding of phosphate groups to metal surfaces (*42*), it seems more likely that this effect is due to the failure of extremely polar phosphorylated peptides to leave the droplet during the electrospray process. Despite this observation, $Ba(OH)_2$ (*32*) treatment in conjunction with ESI ionization was effective

for determining the number of phosphates incorporated into *D*S6A upon mitogenic stimulation.

The S6K1 signaling pathway which leads to ribosomal protein S6 phosphorylation following mitogenic stimulation has been intensively studied in mammalian systems (*43*). The data presented here show that the response of *Drosophila* Kc 167 cells to mitogenic stimulation is very similar to cultured mammalian cells, with the sites of phosphorylation identified as Ser_{233}, Ser_{235}, Ser_{239}, Ser_{242}, and Ser_{245}. In addition, the data in Figure 4 suggest the possible existence of a sixth phosphorylation site; however, this would appear to be a minor component as only five sites were identified by either direct sequencing or phosphate release. Despite the fact that the alignments of the mammalian and *D*S6A sequences containing the phosphorylation sites do not show a clear homology, the overall pattern of phosphorylation appears to be conserved. In fact, in both cases the first two serines following the basic RXR motif are phosphorylated and the last three sites are all separated by an unphosphorylated serine. If one assumes that the pattern of phosphorylation is conserved among insects, then it seems likely that Ser_{237}, Ser_{239}, Ser_{243}, Ser_{246}, and Ser_{249} are the sites of phosphorylation in *Manduca sexta* S6 (Figure 1). The protein kinase responsible for phosphorylating mammalian S6 in vivo has been shown to be S6K1, and more recently S6K2 (*6*). Synthetic peptides containing the RRXS motif are efficiently phosphorylated by S6K1 in vitro (*44*). In analogy to mammalian S6, Ser_{235} in *Drosophila* S6 fits the consensus sequence R(R)RXXSX (*15*), having a basic residue at positions n-3 and n-5. The *D*S6A phosphorylation sites Ser_{242} and Ser_{245} do not have an obvious consensus motif. However, basic residues are found at the n-5 position. It is very likely that secondary and tertiary structural determinants contribute to the recognition and ordered phosphorylation by the *D*S6K.

The phosphorylation of mammalian ribosomal protein S6 is an ordered process, both in vivo and in vitro (*13*, *42*). Two-dimensional TLE analysis of *D*S6A phosphoderivatives digested individually with the endoproteinase LysC also supports an analogous mechanism of phosphorylation for *D*S6A. This is because each increasingly phosphorylated *D*S6A derivative yields a unique set of phosphopeptides, with one particular phosphopeptide increased in intensity in each case. If phosphates were incorporated randomly into all five sites, the phosphopetide pattern would be expected to be similar regardless of the *D*S6A derivative which is analyzed. Attempts to delineate the order of phosphorylation by calculating relative electrophoretic mobilities of individual phosphopeptides (*31*) were not conclusive. Therefore, to precisely determine the order of phosphorylation of *D*S6A, it will be necessary to identify the sites of phosphorylation present in each of the increasingly phosphorylated *D*S6A derivatives (Figure 2A). This will allow direct comparison of the order of phosphorylation between *Drosophila* and mammalian S6. To do so, each of the increasingly phosphorylated derivatives of *D*S6A could be purified and enzymatically digested, and the respective phosphopeptides could be isolated. The sites of phosphorylation in each phosphopeptide could then be determined by protein sequencing after conversion of phosphoserines to their *S*-ethylcysteine derivatives.

In mammals, the synthesis of proteins encoded by 5′TOP mRNAs is thought to be positively regulated by increased S6 phosphorylation mediated by S6K activation (*11*). The 5′TOP mRNAs encode for components of the translational apparatus, most notably ribosomal proteins (*45*). In *Drosophila*, as in mammals, the expression of ribosomal proteins is selectively controlled at the translational level (*46*−*48*). Taking advantage of the ability to generate transgenic flies expressing *D*S6A phosphorylation site mutants, it should be possible to assess whether the effects of S6K are mediated through *D*S6A phosphorylation. In this regard, it should be noted that the gene coding for *D*S6 is made up of three exons; however, two identical alternative tandem repeats exist for exon 3A, termed 3B and 3C (*49*). Alternative splicing has been reported to produce a transcript that would encode the 189 amino acid *D*S6B, which is considerably shorter than the 248 amino acid *D*S6A described here (*39*). Although the existence of mRNA transcripts for the shorter *D*S6B has been reported (*50*), no evidence was obtained here for *D*S6B being associated with ribosomes (Table 1). Nevertheless, it is interesting to note that *DS6* is located on the X chromosome and although hemizygous males and homozygous females harboring a P-element-induced mutation are lethal, the third instar larvae develop a melanotic tumor phenotype (*24*, *25*), leading to the suggestion that *DS6* may serve as a tumor suppressor gene (*23*). These apparently discrepant phenotypes might be reconciled by the existence of a nonribosomal shorter *D*S6B. The results presented here provide a rationale for a detailed analysis of the role of phosphorylation of *D*S6A in *Drosophila*.

ACKNOWLEDGMENT

We thank M. Adler for stimulating discussions and Drs. P. Dennis and S. Fumagalli for their critical reading of the manuscript.

REFERENCES

1. Pardee, A. B. (1989) *Science 246*, 603−608.
2. Krebs, E. G. (1994) *Trends Biochem. Sci. 19*, 439.
3. Brooks, R. F. (1977) *Cell 12*, 311−317.
4. Kozma, S. C., Ferrari, S., and Thomas, G. (1989) *Cell. Signaling 3*, 219−225.
5. Reinhard, C., Fernandez, A., Lamb, N. J. C., and Thomas, G. (1994) *EMBO J. 13*, 1557−1565.
6. Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Kozma, S. C. (1998) *EMBO J. 17*, 6649−6659.
7. Gout, I., Minami, T., Hara, K., Tsujishita, Y., Filinenko, V., Waterfield, M. D., and Yonezawa, K. (1998*) J. Biol. Chem. 273*, 30061−30064.
8. Saitoh, M., ten Dijke, P., Miyazono, K., and Ichijo, H. (1998) *Biochem. Biophys. Res. Commun. 253*, 470−476.
9. Peterson, R. T., and Schreiber, S. L. (1998) *Curr. Biol. 8*, 248−250.
10. Jeffries, H. B. J., Reinhard, C., Kozma, S. C., and Thomas, G. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4441−4445.
11. Jeffries, H. B. J., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. (1997) *EMBO J. 16*, 3693−3704.
12. Nygard, O., and Nilsson, L. (1990) *Eur. J. Biochem. 191*, 1−17.
13. Martin-Pérez, J., and Thomas, G (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 926−930.
14. Krieg, J., Hofsteenge, J., and Thomas, G. (1988) *J. Biol. Chem. 263*, 11473−11477.
15. Flotow, H., and Thomas, G. (1992) *J. Biol. Chem. 267*, 3074−3078.

16. Watson, K. L., Chou, M. M., Blenis, W. M., Gelbart, M. W., and Erickson, R. L. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 13694−13698.
17. Stewart, M. J., Berry, C. O. A., Zilberman, F., Thomas, G., and Kozma, S. C. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 10791−10796.
18. Madjar, J. J., and Fournier, A. (1987) *Eur. J. Biochem. 163*, 577−582.
19. Song, Q., and Gilbert, L. I. (1994) *Dev. Genet. 15*, 332−338.
20. Song, Q., and Gilbert, L. I. (1997) *J. Biol. Chem. 272*, 4429−4435.
21. Kozma, S. C., McGlynn, E., Siegmann, M., Reinhard, C., Ferrari, S., and Thomas, G. (1993) *J. Biol. Chem. 268*, 7134−7138.
22. Olsen, A. S., Triemer, D. F., and Sanders, M. M. (1983) *Mol. Cell. Biol. 3*, 2017−2027.
23. Watson, K. L., Justice, R. W., and Bryant, P. J. (1994) *J. Cell Sci., Suppl. 18*, 19−33.
24. Stewart, M. J., and Denell, R. (1993) *Mol. Cell. Biol. 13*, 2524−2535.
25. Watson, K. L., Konrad, K. D., Woods, D. F., and Bryant, P. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11302−11306.
26. Siegmann, M., and Thomas, G. (1987) *Methods Enzymol. 146*, 362−369.
27. McConkey, E. H., Bielka, H., Gordon, J., Lastick, S. M., Lin, A., Ogata, K., Reboud, J. P., Traugh, J. A., Traut, R. R., Warner, J. R., Welfle, H., and Wool, I. G. (1979) *Mol. Gen. Genet. 169*, 1−6.
28. Nick, H. P., Wettenhall, R. E. H., Hearn, M. T. W., and Morgan, F. J. (1985) *Anal. Biochem. 148*, 93−100.
29. Davis, M. T., and Lee, T. D. (1992) *Protein Sci. 1*, 935−944.
30. Davis, M. T., Stahl, D. C., Hefta, S. A., and Lee, T. L. (1995) *Anal. Chem. 67*, 4549−4556.
31. Boyle, W. J., van der Greer, P., and Hunter, T. (1991) *Methods Enzymol. 201*, 110−149.
32. Byford, M. F. (1991) *Biochem. J. 280*, 261−265.
33. Meyer, H. E., Hoffmann-Posorske, E., and Heilmeyer, L. M. G. (1991) *Methods Enzymol. 201*, 169−185.
34. Spencer, T. A., and Mackie, G. A. (1993) *Biochim. Biophys. Acta 1172*, 332−334.
35. Wettenhall, R. E. H., Aebersold, R. H., and Hood, L. E. (1991) *Methods Enzymol. 201*, 186−199.
36. Olivier, A. R., Ballou, L. M., and Thomas, G. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 4720−4724.
37. Chung, J., Kuo, C. J., Crabtree, G. R., and Blenis, J. (1992) *Cell 69*, 1227−1236.
38. Resing, K. A., Johnson, R. S., and Walsh, K. A. (1995) *Biochemistry 34*, 9477−9487.
39. Stewart, M. J., and Thomas, G. (1994) *Bioessays 16*, 809−815.
40. Schneider, U., Mini, T., Jenö, P., Fisher, P. A., and Stuurman, N. (1999) *Biochemistry 38*, 4620−4632.
41. Payne, M. D., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J.-H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991) *EMBO J. 10*, 885−892.
42. Ferrari, S., Bandi, H. R., Hofsteenge, J., Bussian, B. M., and Thomas, G. (1991) *J. Biol. Chem. 266*, 22770−22775.
43. Pullen, N., and Thomas, G. (1997) *FEBS Lett. 410*, 78−82.
44. Leighton, I. A., Dalby, K. N., Caudwell, F. B., Cohen, P. T. W., and Cohen, P. (1995) *FEBS Lett. 375*, 289−293.
45. Meyuhas, O., Avni, D., and Shama, S. (1996) in *Translational Control of Ribosomal Protein mRNA in Eukaryotes* (Hershey, J. W. B., Mathews, M. B., and Sonenberg, N., Eds.) pp 363−388, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
46. Al-Atia, G. R., Fruscoloni, P., and Jacobs-Lorena, M. (1985) *Biochemistry 24*, 5798−5803.
47. Kay, M. A., and Jacobs-Lorena, M. (1987) *Trends Genet. 3*, 347−351.
48. Patel, R. C., and Jacobs-Lorena, M. (1992) *J. Biol. Chem. 267*, 1159−1164.
49. Stewart, M. J., and Denell, R. (1993) *J. Mol. Biol. Evol. 10*, 1041−1047.
50. Watson, K., Konrad, K., Bryant, P., Erikson, R., and Gelbart, W. (1994) *35th Annual Drosophila Research Conference Program and Abstracts Volume*, p 206.

BI9927484