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Activation of S6 kinase activity in 3T3-L1 cells by insulin and phorbol ester

(phosphorylation/ribosomal proteins/growth factors)

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ABSTRACT **Treatment of 3T3-L1 cells with 0.1–1.0 nM insulin results in rapid (5–15 min) activation of a soluble protein kinase that phosphorylates serine residues in ribosomal protein S6. The insulin-stimulated kinase activity is detectable in confluent, nongrowing preadipocytes and adipocytes. In the presence of 2 μg of cycloheximide per ml, preconfluent 3T3-L1 cells also respond to insulin by acquiring an S6 kinase activity whose properties are the same as those of the enzyme activity elicited by insulin alone in growth-inhibited cells. The principal insulin-stimulated S6 kinase has a M_r of ≈50,000–60,000; there is a variable amount of activity that sediments with a M_r of about 80,000. The soluble enzyme exhibits optimal activity between pH 8 and pH 9, requires Mg^{2+} (10–20 mM), and is inhibited by Ca^{2+} (0.5 mM), Mn^{2+} (0.05 mM), and NaF (30 mM). GTP cannot substitute for ATP in the phosphotransferase reaction; cAMP, cGMP, phosphatidylserine plus diolein, the cAMP-dependent protein kinase inhibitor, and heparin (0.7 μg/ml) are without effect. Although treatment of 3T3-L1 cells with insulin does not influence the activity or the subcellular distribution of the phospholipid and Ca^{2+}-dependent protein kinase C, exposure to the phorbol tumor promoter phorbol 12-myristate 13-acetate (PMA) results in translocation of protein kinase C to the membrane and activation of a soluble phospholipid and Ca^{2+}-independent S6 protein kinase that has the same magnitude of activity and sedimentation behavior as the insulin-induced activity. Trypsin treatment of either 3T3-L1 cytosolic extracts or partially purified 3T3-L1 protein kinase C generates a small amount of S6 kinase activity of M_r 50,000. This activity, resolved by sucrose gradient centrifugation, is less active than that elicited by either insulin or PMA and, unlike the activities generated by insulin and PMA, is associated with histone kinase activity. The data suggest that the S6 kinase elicited by either insulin or PMA is neither protein kinase C, its phospholipid, and Ca^{2+}-independent proteolytic derivative nor the result of proteolytic activation of an inactive proenzyme that can be reproduced by trypsin treatment of cell extracts *in vitro*.**

Multisite phosphorylation of ribosomal protein S6 in cultured cells occurs in response to the addition of serum (1–3), hormones and growth factors (including insulin) (4–9), and phorbol esters (10, 11). *In vitro*, S6 is a substrate for several different protein kinases, including cAMP- and cGMP-dependent protein kinases (12–15), rat brain phospholipid and Ca^{2+}-dependent kinase (16), lymphocyte H4 kinase (17), protease-activated kinases (PAK II) from reticulocytes (18), liver (19), and 3T3-L1 cells (5), and a membrane-associated protein kinase from 3T3-L1 cells (20). A dramatic increase in soluble S6 kinase activity has recently been reported to occur in serum-treated quiescent fibroblasts (3). This activation, measured in cell extracts, was stabilized by buffers containing EGTA and β-glycerophosphate, suggesting the possibility that modification of the enzyme by phosphorylation might be involved in its activation.

We now describe a soluble, insulin- and phorbol ester-stimulated S6 protein kinase activity in 3T3-L1 cells that does not appear to be the same as any of the purified enzymes previously reported to catalyze the phosphorylation of ribosomal protein S6. The enzyme is activated within minutes of exposure of differentiated 3T3-L1 adipocytes to nanomolar concentrations of insulin or the phorbol ester tumor promoter phorbol 12-myristate 13-acetate (PMA).

EXPERIMENTAL PROCEDURES

Materials. Histone (type III S), cycloheximide, bacitracin, pepstatin, aprotinin, leupeptin, and soybean trypsin inhibitor were from Sigma. Dithiothreitol was from Calbiochem. Trypsin (treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone) was from Worthington. PMA was from P-L Biochemicals. Porcine insulin was obtained from Eli Lilly (M. Root). αS_1-Casein was provided by E. Bingham (U.S. Dept. of Agriculture, Philadelphia). Purified eukaryotic initiation factor 2 (eIF2) was provided by U. Maitra (Albert Einstein College of Medicine). The protein inhibitor of cAMP-dependent protein kinase was a gift from J. DeMaille (Institut National de la Santé et de la Recherche Médicale, Montpellier, France). [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) was from Amersham. The 40S ribosomal subunit was prepared from *Artemia salina* according to the method of Zasloff and Ochoa (21).

Cell Culture and Preparation of Cell Extracts. 3T3-L1 cells were grown to confluence (day 0) and stimulated to differentiate as described (22). Prior to insulin treatment, either preconfluent or confluent preadipocytes or differentiated 3T3-L1 adipocytes were incubated in Krebs–Ringer bicarbonate/Hepes buffer (120 mM NaCl/4.75 mM KCl/1.2 mM $MgSO_4$/1.2 mM $CaCl_2$/24 mM $NaHCO_3$/10 mM Hepes, pH 7.5) containing 2% dialyzed bovine serum albumin (hereafter referred to as KRB/albumin buffer) for 1 hr (4). Insulin (at the indicated concentration) was added directly to the medium. Following hormone treatment, media were aspirated and the cells were washed with 15 ml of a solution of 80 mM β-glycerophosphate, pH 7.4/20 mM EGTA/15 mM $MgCl_2$/aprotinin at 25 μg/ml/pepstatin at 10 μg/ml/leupeptin at 10 μg/ml/soybean trypsin inhibitor at 10 μg/ml. Cells were homogenized in this buffer with 100 strokes of a Dounce homogenizer. The homogenate was first centrifuged at 400 × *g* for 5 min to remove nuclei and unbroken cells and then at 100,000 × *g* for 1 hr. Fractions either were used immediately or were stored at −70°C. For experiments designed to examine trypsin-generated S6

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Abbreviations: eIF, eukaryotic initiation factor; Me_2SO, dimethyl sulfoxide; PMA, phorbol 12-myristate 13-acetate.

kinase, protease inhibitors were omitted from the homogenization buffer.

Enzyme Assays. Protein kinase assays were routinely performed for 15 min at 30°C in a final volume of 30 μl containing 20 mM Hepes buffer (pH 7.4), 8 mM $MgCl_2$, 2 mM dithiothreitol, and 100 μM [γ-^{32}P]ATP (8 cpm/fmol) in the presence of either 0.3–1.5 mg of 40S *Artemia* ribosomal subunits per ml, 1 mg of histone per ml, 1 mg of αS_1-casein per ml, or 0.6 mg of eIF2 per ml. Reactions were terminated by addition of 8 μl of $NaDodSO_4$ sample buffer (10% $NaDodSO_4$/0.3 M Tris·HCl, pH 6.8/50% glycerol) and aliquots were analyzed by $NaDodSO_4$ gel electrophoresis on 15% polyacrylamide gels (4). The gels were stained with Coomassie brilliant blue, dried, and autoradiographed on Kodak XAR film. The protein band(s) corresponding to either S6, histone, or casein was excised from the dried gels and the ^{32}P incorporated was quantitated by liquid scintillation spectroscopy.

Phospholipid and Ca^{2+}-dependent protein kinase activity was assayed as described (16). The reaction was carried out for 15 min in 40 μl containing 20 mM Hepes buffer (pH 7.4), 8 mM $MgCl_2$, 2 mM dithiothreitol, 80 μg of phosphatidylserine per ml, 8 μg of diolein per ml, 1.2 mM $CaCl_2$ (in excess over the 2.5 mM EGTA contributed by addition of enzyme), 1 mg of histone (type III) per ml, and 120 μM [γ-^{32}P]ATP (0.5–1.0 cpm/fmol). Control reactions lacked added phospholipid, diolein, and Ca^{2+}. Protein kinase C was converted to protein kinase M (23) by treatment with trypsin (4 μg of trypsin per 200-μl extract made to 1 mg of protein per ml by the addition of bovine serum albumin) for 3 min at 30°C. Proteolysis was terminated by the addition of soybean trypsin inhibitor (400 μg/ml). Conversion was quantitated by determining units of phospholipid and Ca^{2+}-dependent histone kinase activity converted to units of phospholipid and Ca^{2+}-independent histone kinase activity.

Other Methods. Calculations of molecular weights by sucrose density gradient ultracentrifugation were performed as described by Martin and Ames (24). Centrifugation was performed by using a TH 641 swinging bucket rotor (Beckman) at 36,000 rpm for 18 hr at 4°C. Linear gradients (10 ml, 5–20% sucrose) were prepared in 40 mM β-glycerophosphate buffer (pH 7.3) containing 10 mM EGTA and 8 mM $MgCl_2$. Cell extracts, together with marker proteins ovalbumin ($s_{20,w}$ = 3.5) and bovine serum albumin ($s_{20,w}$ = 4.3), were applied to each gradient in a total volume of 200–300 μl. Protein was determined by the Coomassie blue technique (25) using concentrated protein reagent from Bio-Rad. Phosphoamino acid analysis of S6 was performed as described (20).

RESULTS AND DISCUSSION

Insulin-Stimulated S6 Kinase Activity. Treatment of confluent 3T3-L1 preadipocytes or adipocytes with 1 μM insulin results in a 5- to 10-fold increase in S6 protein kinase activity (Fig. 1, lanes 2 and 6). In contrast, growing (preconfluent) preadipocytes did not show a significant increase in S6 kinase activity in response to insulin. The inclusion of cycloheximide (2 μg/ml) for 30 min prior to (and during) exposure to insulin potentiated the activation of S6 kinase in quiescent adipocytes and preadipocytes (Fig. 1, lanes 4 and 8) and permitted expression of insulin-stimulated S6 kinase in preconfluent cells (data not shown). Since treatment of 3T3-L1 adipocytes with cycloheximide under these conditions inhibits protein synthesis by >90% (26) it is likely that insulin activates a preexisting protein kinase. The data also suggest that the synthesis or turnover of some other protein(s) may be involved in this activation.

Stimulation of S6 kinase activity was detected as early as 5 min after the addition of insulin to 3T3-L1 adipocytes and was half-maximal at 15 min (Fig. 2*A*). Stimulation was

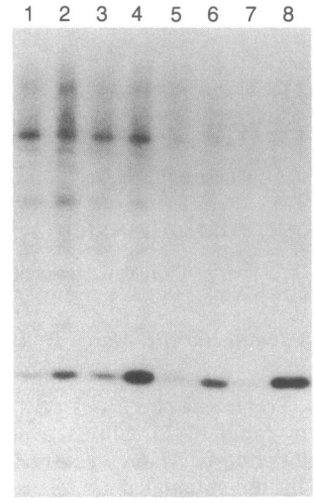

FIG. 1. Activation of S6 kinase by insulin. Cells were washed three times with KRB/albumin buffer and then incubated in this medium for 1 hr. Where indicated (lanes 3, 4, 7, and 8), 2 μg of cycloheximide per ml was added during the last 30 min of preincubation with KRB/albumin buffer. Confluent preadipocytes (day 6) (lanes 1–4) and adipocytes (day 6) (lanes 5–8) were treated with (lanes 2, 4, 6, and 8) or without (lanes 1, 3, 5, and 7) 1 μM insulin for 60 min. Cells were harvested and cytosolic extracts were prepared. Aliquots (40 μg of protein) of each extract were assayed at 30°C in a total volume of 30 μl containing 20 mM Hepes buffer (pH 7.4), 8 mM $MgCl_2$, 2 mM dithiothreitol, 8 μg of 40S ribosomal proteins, and 120 μM ATP (8 cpm/fmol) for 10 min. The reactions were terminated by the addition of 8 μl of $NaDodSO_4$ sample buffer. Shown is an autoradiogram following $NaDodSO_4$/PAGE. Films were developed for 3 hr at 20°C with a Dupont Cronex intensifying screen. The arrow points to the position of S6.

apparent at 10 pM insulin and half-maximal at 1 nM insulin (Fig. 2*B*). Thus, physiologic concentrations of insulin can rapidly activate this kinase in quiescent 3T3-L1 cells. As first reported by Novak-Hofer and Thomas (3) for a serum-activated S6 kinase demonstrated in cytosolic extracts of quiescent Swiss 3T3 cells, the insulin-stimulated S6 kinase activity in 3T3-L1 cells was more stable when extracted in a β-glycerophosphate/EGTA buffer than in Hepes or Tris

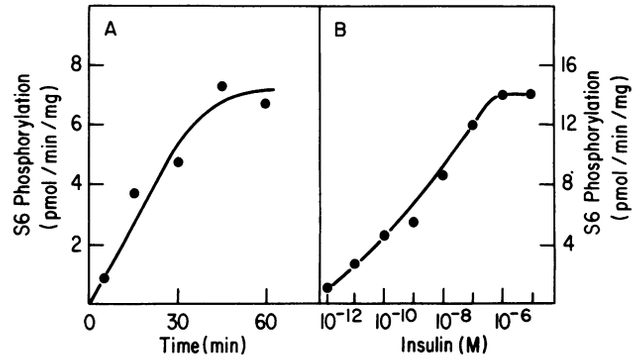

FIG. 2. Time course and dose–response curve for insulin-stimulated S6 kinase activity. (*A*) Adipocytes (day 6) were incubated with KRB/albumin buffer for 1 hr and then treated with or without 1 μM insulin in the presence of 1.5 mM bacitracin (22). At the indicated times, cells were harvested and cytosolic extracts were prepared. Extracts (40 μg of protein) were assayed for 5 min as described in the legend to Fig. 1. Following autoradiography, S6 bands were excised and ^{32}P incorporation was quantitated by liquid scintillation spectroscopy. Data are presented as the difference in pmol of phosphate incorporated into S6 in the presence and absence of insulin. (*B*) Adipocytes (day 6) were incubated as above and then treated with insulin in the presence of 1.5 mM bacitracin for 1 hr. Cells were harvested and assayed as described above.

buffers. Following sucrose gradient centrifugation, neither β-glycerophosphate nor EGTA was required for activity.

Like insulin, phorbol ester tumor promoters induce multisite phosphorylation of ribosomal protein S6 in intact cells (10, 11). Since phorbol esters bind to and activate protein kinase C (27–31), it could be proposed that the effects of insulin, like those of phorbols, might be directly mediated by the action of protein kinase C on S6. In an effort to resolve the insulin-stimulated S6 kinase from protein kinase C, the 100,000 × *g* supernatant fluids from insulin-treated and control cell extracts were subjected to sucrose density gradient ultracentrifugation. As shown in Fig. 3*A*, there is a broad peak of S6 kinase activity that is increased in response to insulin (Fig. 3*B*). The activity sedimenting between M_r 50,000 and M_r 60,000 is consistently stimulated 5- to 10-fold by insulin and, for this reason, is referred to as the insulin-stimulated S6 kinase. The activity that sediments with a M_r of about 80,000 is less consistently elevated. It is not known whether the apparent heterogeneity of activated S6 kinase reflects the presence of more than one enzyme or various forms of one enzyme species. The activity sedimenting in the same position as protein kinase C (see Fig. 3) may reflect basal protein kinase C activity towards S6. However, under the conditions of these experiments (Fig. 3 *C* and *D*) insulin does not change the overall histone kinase activity of protein kinase C. The principal insulin-stimulated S6 kinase that sediments between ovalbumin and bovine serum albumin (Fig. 3*B*) is not stimulated by the addition of phospholipids and Ca^{2+}. An additional feature of 3T3-L1 protein kinase C that can be used to distinguish it from the insulin-activated enzyme is that the former catalyzes the phosphorylation of another 40S associated protein (M_r = 17,000), whereas the insulin-activated enzyme does not (see Fig. 4).

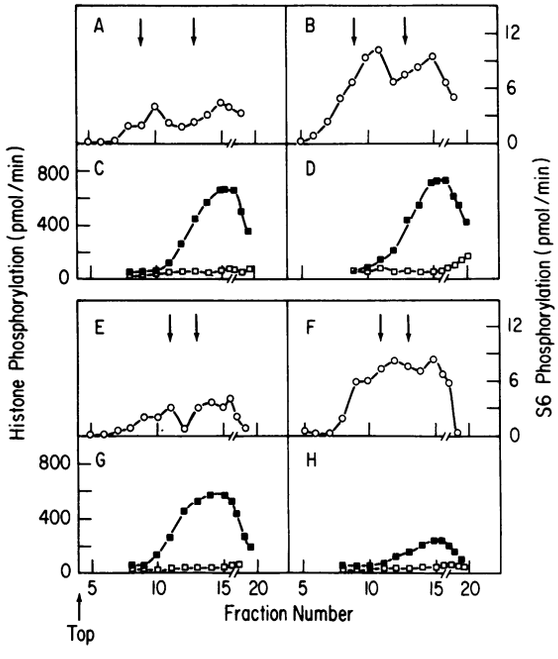

FIG. 3. Analysis of insulin- and PMA-stimulated S6 kinase activity by sucrose density gradient ultracentrifugation. Adipocytes (day 6) were washed three times with KRB/albumin buffer and incubated in this medium for 1 hr. Cells were then treated with (*B* and *D*) or without (*A* and *C*) 1 μM insulin or with dimethyl sulfoxide (Me_2SO) (*E* and *G*) or PMA (200 nM) (*F* and *H*) for 1 hr. Cells were harvested and processed. Extracts (200–300 μl) derived from 2 × 10^6 cells were applied to 10-ml linear sucrose gradients (5–20%). Following centrifugation, 50 fractions of 200 μl each were collected. Aliquots (10 μl) of the indicated fractions were assayed either for S6 kinase activity (○) or protein kinase C activity in the absence (□) or presence (■) of phospholipids and Ca^{2+}. The two arrows (from left to right) indicate the sedimentation positions of marker proteins, ovalbumin and bovine serum albumin, respectively.

Protease-Stimulated S6 Kinase Activity. Protease-activated kinase II (PAK II), an enzyme isolated from reticulocytes (18) and liver (19), is a phospholipid and Ca^{2+}-independent protein kinase that, when activated by trypsin, catalyzes the phosphorylation of S6 (18) as well as eIF2 and histone (32). The inactive proenzyme has a M_r of about 80,000 and, in its activated form, has a M_r of 50,000–60,000. To determine whether the observed insulin-stimulated S6 kinase was derived from a proenzyme of this kind, sucrose gradient fractions in the M_r region of 60,000–90,000 from control cells were pooled and divided into two portions. One aliquot was treated with trypsin (4 μg of trypsin for 3 min per 200 μg of protein), whereas the other portion was not. Following the addition of soybean trypsin inhibitor, both were then resedimented through sucrose gradients. Proteolysis resulted in decreased protein kinase C activity and concomitant appearance of a phospholipid and Ca^{2+}-independent histone kinase activity designated protein kinase M by Castagna *et al.* (27) (Fig. 4). This conversion was accompanied by the appearance of a small amount of phospholipid and Ca^{2+}-independent S6 kinase activity (about 10–20% that observed after exposure to insulin) in the same position (M_r = 50,000) as protein kinase M. The protease-generated activity or activities also phosphorylated the ribosome-associated protein of M_r = 17,000, which is a good substrate for protein kinase C but does not appear to be a substrate for either the insulin- or PMA-activated S6 kinase (see Fig. 4). When the 100,000 × *g* supernatant fluids derived from homogenates of either insulin-treated or control cells were treated directly with trypsin (4 μg of trypsin per 3 mg of extract protein for 3 min at 30°C) there was a similar disappearance of protein kinase C, a concomitant increase in phospholipid and Ca^{2+}-independent histone kinase activity (protein kinase M), and a similarly small increase (compared to that elicited by insulin) in Ca^{2+} and phospholipid-independent S6 kinase activity. Trypsinization for shorter or longer periods of time failed to generate more S6 kinase. These data indicate that trypsin treatment of either proteins of M_r 60,000–90,000 or unfractionated 3T3-L1 cytosolic extracts results in the quantitative conversion of protein kinase C to protein kinase M. The S6 kinase activity generated by proteolysis, which may be protein kinase M and/or some other protein kinase of

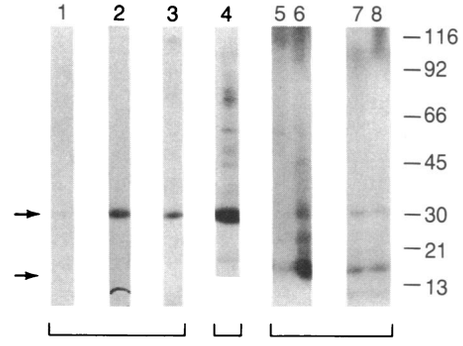

FIG. 4. S6 phosphorylation by protein kinases isolated from sucrose gradients. Gradient fractions containing the peak S6 kinase activity (M_r ≈50,000) derived from control (lane 1) or insulin- (lane 2) or PMA-treated (lane 3) confluent 3T3-L1 adipocytes were pooled and reassayed for S6 kinase activity. In a separate experiment, the peak fractions of S6 kinase activity (M_r ≈50,000) from insulin- plus cycloheximide-treated (lane 4) cells were assayed for S6 kinase activity. In addition, protein kinase C and protein kinase M derived by treating the 3T3-L1 protein kinase C-containing fractions with trypsin were resedimented on 5–20% sucrose gradients and then reassayed for S6 kinase activity in the absence (lanes 5 and 7) or presence (lanes 6 and 8) of phospholipids and Ca^{2+}. Arrows indicate the positions of S6 and of the M_r = 17,000 species.

similar molecular weight, is much less than that found in extracts derived from cells treated with insulin.

PMA-Stimulated S6 Kinase. When extracts from Me_2SO-treated (control) or PMA-treated cells were sedimented in sucrose gradients (Fig. 3 *E–H*), an increase in S6 kinase activity was observed. In contrast to insulin, treatment with PMA led to a 70% decrease in cytosolic protein kinase C activity (Fig. 3 *G* and *H*) [as described previously for other cell types (29, 33)] accompanied by an increase in protein kinase C associated with the particulate fraction of the cell (not shown). There was no evidence for generation of either membrane-associated or cytosolic protein kinase M. It should be noted with respect to Fig. 3, that although only the first 20 of the 50 fractions collected from each gradient are depicted, entire gradients were assayed for S6 kinase, protein kinase C, and trypsin-activated S6 kinase activities and none of these activities was found in later fractions.

Resolution of the Activated S6 Kinases. To distinguish among the S6 protein kinase activities elicited by insulin, PMA, and limited proteolysis and to resolve them from protein kinase C, gradient fractions containing these activities (M_r 50,000–60,000) were individually pooled, concentrated, and resedimented on sucrose gradients. The fractions containing the S6 kinase (which resedimented at the same position as the applied kinase) were then pooled, concentrated, and tested for ability to catalyze the phosphorylation of S6, histone, eIF2, and casein. Of the substrates tested, the insulin-generated kinase exhibited an increased activity toward S6 alone. In contrast, the protein kinase(s) generated by trypsin catalyzed the phosphorylation of histone, eIF2, and the 40S ribosomal protein of M_r 17,000 at least as well as S6. The PMA-generated enzyme as well as the enzyme generated in preconfluent cells by treatment with insulin and cycloheximide shared the substrate specificity pattern of the insulin-stimulated S6 kinase. Although these results do not exclude a role for a protease-activated protein kinase in insulin-promoted S6 kinase activation, they do indicate that the principal effect of insulin or PMA in eliciting the soluble S6 kinase activity described herein cannot be mimicked by limited proteolysis of cell extracts and is not due to either unmodified protein kinase C or protein kinase M.

Properties of the Insulin-Stimulated S6 Kinase. The insulin-stimulated S6 kinase was optimally active between pH 8 and pH 9. Enzymic activity was not altered by the addition of cAMP (5 μM), cGMP (5 μM), phosphatidylserine plus diolein (80 μg/ml and 8 μg/ml, respectively), or heparin (0.7 μg/ml). NaF (30 mM) completely inhibited activity. The protein inhibitor of cAMP-dependent protein kinase (34) at concentrations sufficient to inhibit 10 pmol of ^{32}P transferred to histone per minute by cAMP-dependent protein kinase had no effect on the insulin-stimulated S6 kinase.

The dialyzed enzyme was found to require Mg^{2+} (10–20 mM) and ATP for activity; GTP was not able to substitute for ATP. The enzyme was inhibited 50% by Mn^{2+} (0.05 mM) or Ca^{2+} (0.5 mM). Thus, the insulin-stimulated S6 kinase is both cyclic nucleotide- as well as phospholipid- and Ca^{2+}-independent. Mixtures of high-speed supernatant fluids derived from insulin-stimulated and control cells yielded activities that were additive. Phosphorylation of S6 by the insulin- or PMA-stimulated S6 kinase occurred on serine residues (see *Experimental Procedures*). It is not known, however, which of the sites on S6 become phosphorylated. The insulin-stimulated S6 kinase is 90% soluble; the 10% present in the particulate fraction has the same properties as the soluble enzyme.

In conclusion, physiological concentrations of insulin rapidly activate an S6 protein kinase in 3T3-L1 cells. The activity is soluble, sediments as a broad peak in sucrose gradients, and is neither cAMP- nor phospholipid- and Ca^{2+}-dependent. An activity of similar sedimentation behavior and substrate specificity is elicited by PMA. The properties of the insulin-stimulated S6 kinase distinguish it from cAMP- and cGMP-dependent protein kinases, protein kinases C and M, casein kinases, and protein kinases that can be activated, *in vitro*, by limited proteolysis with trypsin. Although the mechanism by which activation occurs is not known, it is unlikely to result from the direct phosphorylation of a less active form of the S6 kinase by both the insulin-dependent tyrosine protein kinase and protein kinase C since these enzymes have dissimilar catalytic activities. It is possible that activation of tyrosine protein kinases (including the insulin receptor) and protein kinase C trigger discrete biochemical pathways that ultimately converge to generate the same S6 protein kinase.

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