Differential Stimulation of Phosphorylation of Initiation Factors eIF-4F, eIF-4B, eIF-3, and Ribosomal Protein S6 by Insulin and Phorbol Esters*

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Protein phosphorylation is a fundamental regulatory mechanism involved in the control of cellular processes by hormones. Treatment of cells with insulin causes specific changes in the phosphorylation of a number of cellular proteins as a result of insulin-induced alterations in the activity of specific protein kinases and/or phosphoprotein phosphatases (1–3). Changes in the phosphorylation state of key enzymes mediates metabolic responses, including alterations in translation (4, 5). A number of different protein kinases appear to be involved in the insulin response, including the tyrosine protein kinase of the insulin receptor, serine/threonine protein kinases modulating ribosomal protein S6 (4, 6), and microtubule-associated protein-2 kinase (1, 4, 7, 8). Other hormones involved in growth and differentiation mediate changes in cellular metabolism by activation of the Ca2+/phospholipid-dependent protein kinase (9, 10). Protein kinase C is regulated by modulation of diolien levels through activation of phospholipase C. The enzyme also serves as a receptor for PMA, which substitutes for diolien in the activation process.

One of the initial events occurring in response to stimulation with insulin or PMA is the multiple phosphorylation of ribosomal protein S6 (4, 11). The same sites are modified in vivo in response to a number of mitogenic compounds, including PMA and insulin. To date, four translational initiation factors, 2, 3, 4B, and 4F have been shown to be phosphorylated in vivo (12–16, 19). Phosphorylation of eIF-3 p170, eIF-4B, and eIF-4F p25 and p220 is stimulated in reticulocytes in response to PMA (15). eIF-4B and eIF-4E/eIF-4F p25 have also been shown to be dephosphorylated upon heat shock, and during mitosis or serum-starvation of exponentially growing cells (13, 14, 19).

All four of the phosphorylated initiation factors play a crucial role in the translation process (20–22). eIF-2, which has been studied extensively, is involved in the binding of initiator tRNA to the 40 S preinitiation complex (20). eIF-4F is required for the efficient translation of capped mRNA (22–24) and is a major discriminatory factor in protein synthesis initiation (25). The p25 subunit is known to specifically recognize the mRNA cap structure (16, 20), while the function of p220, a critical component of eIF-4F (23, 27–29), remains unclear. It has been suggested that p220 is involved in the alignment of eIF-4A with the mRNA cap and p25 (21). eIF-3 and eIF-4B often co-purify with eIF-4F (15, 18, 30). Although they are not stable components of the cap-binding protein complex, they associate with eIF-4F during the initiation process (30). eIF-3 is required for mRNA binding to the 43 S initiation complex (31, 32) and has ribosomal subunit dissociation activity (33). eIF-4B is involved in the recycling of eIF-4F between successive mRNAs (21, 30, 34, 35).

Previously, Tuazon et al. (36) have shown that eIF-3, eIF-4F, and eIF-4A are phosphorylated in vitro by at least three multipotential protein kinases. eIF-4F p25 and p220 are modified by protein kinase C at the same sites modular in vivo.
in response to PMA (15). Protease-activated kinase II, implicated in the insulin response (37), modifies the p220 subunit in vitro (38). In these studies, we utilized eIF-4F to examine differences in the metabolic activation of protein kinases in response to insulin and PMA. At the same time, phosphorylation of eIF-3 and eIF-4B were examined, due to association of these factors with eIF-4F, and the data correlated with phosphorylation of S6. We found that phosphorylation of initiation factors in response to insulin and PMA is mediated by PMA-dependent and insulin-dependent pathways, the latter of which is mediated, at least in part, through protease-activated kinase II.

EXPERIMENTAL PROCEDURES

Materials—[32P]Orthophosphate was obtained from ICN Radiochemicals; porcine insulin was from Eli Lilly; trypsin (dipeptidylcarboxyl chloride-treated) and PMA were from Sigma. m7GTP-Sepharose 4B and m7GTP were purchased from Pharmacia LKB Biotechnology Inc. Thin layer cellulose sheets were from Eastman Kodak. Purified initiation factors were kindly provided by Dr. W. C. Merrick (Case Western Reserve University School of Medicine).

Labeling of 3T3-L1 Cells with [32P]Orthophosphate—3T3-L1 cells were grown to confluence as described previously (37) and washed twice with phosphate-free Dulbecco’s modified Eagle’s medium. Cells were then incubated in the same medium with [32P]orthophosphate (2 mCi/ml) in the absence of serum for 1.5 h. At that time, either insulin in 1 mM HCl or 1 mM HCl alone was added; the cells were incubated further at 37 °C as indicated in the figure legends. For PMA treatment, cells were incubated with 1 x 10-7 M PMA in dimethyl sulfoxide or dimethyl sulfoxide alone (15), at 37 °C, for the times indicated. Cell lysates were prepared as described (37) in the presence of phosphatase and protease inhibitors (50 mM NaF, 1 mM benzamide, and 2 mM benzamidine, and antipain), and 32P-labeled eIF-4F was isolated immediately by affinity chromatography on m7GTP-Sepharose, as described by Morley and Traugh (15). Proteolyzed phosphorylated proteins were analyzed by gel electrophoresis (15). Protein was stained with Coomassie Blue and the radioactivity was quantified by scanning the autoradiograms at 660 nm with a transmission densitometer.

Two-dimensional Phosphopeptide Mapping of eIF-4F—Following polyacrylamide gel electrophoresis, individual subunits of eIF-4F were excised from the gel and subjected to trypsin digestion and two-dimensional phosphopeptide mapping as described (15, 36). The phosphopeptides were detected by autoradiography.

RESULTS

Stimulation of Phosphorylation of eIF-4F in Quiescent 3T3-L1 Cells in Response to Insulin—To examine whether the phosphorylation state of eIF-4F in quiescent, serum-starved 3T3-L1 cells was changed in response to insulin, cells were incubated with [32P]orthophosphate in the absence of serum for 1.5 h, and then incubated in the absence or presence of 10-7 M insulin for 1.5 h. eIF-4F was isolated by m7GTP-Sepharose chromatography in the presence of protease and phosphatase inhibitors and equal amounts of protein analyzed by polyacrylamide gel electrophoresis. Little incorporation of 32P into eIF-4F was obtained in the absence of insulin (Fig. 1A). However, upon insulin stimulation a large increase in phosphorylation of both p25 and p220 was observed. As shown in the autoradiograms in Fig. 1A, all of the eIF-4F adhered to the column. In agreement with data from studies on this initiation factor from reticulocytes (15, 38), changes in phosphorylation of p25 and p220 had no effect on the binding and recovery of eIF-4E and eIF-4F from m7GTP-Sepharose. Phosphoamino acid analysis of both p25 and p220 showed the presence of phosphoserine (data not shown). The increase in phosphorylation in response to insulin was 2.5-5-fold for p220 and 25, although stimulation as high as 10-fold was seen with the p220 subunit. This coincided with a 2.5-3-fold increase in phosphorylation of ribosomal protein S6 (data not shown). Identical results were obtained when cells were washed prior to the addition of insulin to remove the radio-label. Identical results were also obtained following a 24-h preincubation with 32P, with or without cell washing prior to the addition of insulin. These controls rule out any effect of 32P transport on the insulin- or PMA-induced phosphorylation observed.

To determine whether stimulation of phosphorylation was physiologically relevant, cells were incubated with insulin at concentrations from 10-10 to 10-7 M and 32P-labeled eIF-4F

![Fig. 1](http://www.jbc.org/)

**Fig. 1. Stimulation of phosphorylation of eIF-4F in quiescent 3T3-L1 cells in response to insulin. A,** quiescent 3T3-L1 cells were incubated with [32P]orthophosphate in the absence of serum for 1.5 h and then incubated in the absence (lanes 1–3) or presence (lanes 4–6) of 10-7 M insulin for a further 1.5 h. Cell extracts were prepared and [32P]-labeled eIF-4F was isolated by chromatography on m7GTP-Sepharose. Equal amounts of protein were analyzed by gel electrophoresis as described under "Experimental Procedures"; the resultant autoradiogram is shown. Lanes 1 and 4, cell lysate; lanes 2 and 3, protein which did not adhere to m7GTP-Sepharose; lanes 4 and 5, protein eluted from m7GTP-Sepharose with 0.075 mM m7GTP. Arrows indicate the position of eIF-4F p25 and p220, eIF-4B, and eIF-3 p120. These data are representative of results obtained in six separate experiments. **B,** 3T3-L1 cells were preincubated with [32P] orthophosphate, and then incubated with the indicated final concentrations of insulin for 1.5 h. [32P]-Labeled eIF-4F was isolated and analyzed as described in A. Radioactivity was quantified by scanning the autoradiogram at 660 nm with a transmission densitometer.
was isolated and analyzed. As shown in Fig. 1B, optimal stimulation of phosphorylation of both the p220 and p25 subunits of eIF-4F was observed at 10^{-8} M insulin. This indicates that the stimulation was dependent on physiological concentrations of insulin. In some experiments, an apparent increased level of phosphorylation of eIF-4F p25, as shown in these data, reflects a higher level of phosphorylation during the preincubation period. A similar effect has been reported in studies utilizing reticulocytes (15).

Phosphorylation of eIF-3 and eIF-4B in Response to Insulin—By virtue of association with eIF-4F during the initiation process (30), additional 32P-labeled proteins (5–10% of the total protein) were recovered with eIF-4F during chromatography on m'GTP-Sepharose. As judged by co-migration with purified eIF-3 and eIF-4B upon gel electrophoresis, these phosphoproteins were identified as eIF-3 p120, and eIF-4B. Upon insulin stimulation of cells, a 2–4-fold increase in the phosphorylation of both eIF-4B and eIF-3 p120 was observed at physiologically relevant insulin concentrations (Fig. 1). Phosphoamino acid analysis of eIF-4B and eIF-3 p120 showed only the presence of phosphoserine (data not shown).

Stimulation of Phosphorylation of eIF-4F, eIF-4B, and eIF-3 in Response to PMA—To determine whether PMA could influence the phosphorylation state of eIF-4F in quiescent 3T3-L1 cells, the cells were preincubated with [32P]orthophosphate in the absence of serum and then incubated further for 45 min in the absence or presence of 1 x 10^{-6} M PMA. In the absence of PMA, low levels of phosphorylation of eIF-4F were seen (Fig. 2). Upon PMA stimulation, an increase in phosphorylation of p25 and p220 of 4–5-fold was observed. In addition, a 2–3-fold increase in phosphorylation of eIF-4B and eIF-3 p120 (Fig. 2) was detected in the PMA-stimulated cells. The amounts of eIF-3 and eIF-4B associated with eIF-4F during chromatography on m'GTP-Sepharose were similar, if not identical, from cells incubated in the presence or absence of insulin or PMA. Thus, under all conditions examined, complexation of the factors was not altered by the phosphorylation state.

Two-dimensional Phosphopeptide Maps of p25 and p220—Radioactive subunits of eIF-4F were isolated from control and insulin-stimulated or PMA-treated 3T3-L1 cells, subjected to extensive trypsin digestion and analyzed by two-dimensional phosphopeptide mapping followed by autoradiography. Analysis of p25 from quiescent and insulin-stimulated cells showed a single phosphopeptide (Fig. 3, A and B), the intensity of which was increased with insulin (Fig. 3B). Similarly, a single highly phosphorylated peptide was observed with PMA-treatment (Fig. 3C). This phosphopeptide was identical to that observed upon PMA-induced phosphorylation of p25 in reticulocytes and by phosphorylation of purified eIF-4F in vitro with protein kinase C (15). eIF-4F p220 from quiescent, insulin- or PMA-stimulated cells, showed a more complex phosphopeptide pattern upon trypsin digestion. Analysis of eIF-4F p220 isolated from insulin-stimulated cells showed that, relative to quiescent cells, the intensity of existing phosphopeptides was increased in addition to the presence of new labeled species (Fig. 3, D and E). Upon PMA treatment, similar analyses of p220 showed a phosphopeptide pattern distinct from that obtained with insulin-stimulated cells (Fig. 3F). However, this pattern was very similar to that obtained from p220 isolated from PMA-stimulated reticulocytes (15) and from p220 phosphorylated by protein kinase C in vitro (36).

Stimulation of Phosphorylation of Initiation Factors in Cells Down-regulated with Phorbol Esters—Total cellular protein kinase C activity can be down-regulated by prolonged exposure of cells to phorbol esters (9). These protein kinase C-depleted cells were used to determine whether protein kinase C was the sole mediator of phosphorylation of eIF-4F in vivo. Quiescent cells were treated with 1 x 10^{-6} M PMA or 10^{-7} M insulin for 37 °C for 24 h and then incubated with [32P]orthophosphate for 1.5 h in the absence of serum. Cells were then challenged with 1 x 10^{-6} M PMA or 10^{-7} M insulin for 45 min; following lysis, 32P-labeled eIF-4F was isolated immediately by m'GTP-Sepharose affinity chromatography. As shown in Fig. 4A, addition of fresh PMA led to little or no change in the level of phosphorylation of p25 or p220. This indicates that the cells were deficient in protein kinase C. Similarly, little stimulation
of phosphorylation of eIF-4B or eIF-3 p120 was detected. This was in contrast to the results presented in Fig. 2, where a 4-fold stimulation of phosphorylation of p25 and p220, and a 2-2.5-fold stimulation of phosphorylation of eIF-4B and eIF-3 p120 was observed in direct response to PMA.

When the down-regulated cells were stimulated with insulin, there was little or no change in the level of phosphorylation of eIF-4F p25. However, when incubated with 10^{-8} M PMA, the phosphorylation of both subunits is observed as high as 10-fold has been seen for the p220 subunit. Optimal stimulation of phosphorylation of both subunits is observed at 10^{-8} M insulin, indicating the response is dependent upon physiologically relevant insulin concentrations. Cell extracts prepared from insulin-stimulated cells retain the ability to phosphorylate eIF-4F in vitro (data not shown). Phosphoamino acid analysis of p25 and p220 isolated from insulin-stimulated cells shows only phosphoserine, even when extracts are prepared in the presence of 1 mM vanadate (data not shown). In addition to eIF-4F, other proteins involved in the mRNA binding step of protein synthesis initiation are phosphorylated in response to insulin. Exposure of 3T3-L1 cells to insulin promotes the phosphorylation of eIF-4B, eIF-3 p120, and ribosomal protein S6, typically with a 2.5-3-fold increase in phosphorylation (summarized in Table I). In studies with reticulocytes, purification of eIF-4B and eIF-3 have suggested that the increased phosphorylation observed reflects the total cellular pools of these factors (data not shown).

Treatment of 3T3-L1 cells with 10^{-8} M PMA promotes the phosphorylation of eIF-4F p25 and p220 by 4-fold, with a 2-2.5-fold increase in phosphorylation of eIF-4B and eIF-3.

**DISCUSSION**

We have demonstrated that two subunits of eIF-4F (p25 and p220) are phosphorylated in 3T3-L1 cells and the phosphorylation is stimulated in response to insulin. Phosphorylation of both the p25 and p220 subunits of eIF-4F is stimulated typically 2.5-5-fold, respectively, although stimulation as high as 10-fold has been seen for the p220 subunit. Optimal stimulation of phosphorylation of both subunits is observed at 10^{-8} M insulin, indicating the response is dependent upon physiologically relevant insulin concentrations. Cell extracts prepared from insulin-stimulated cells retain the ability to phosphorylate eIF-4F in vitro (data not shown). Phosphoamino acid analysis of p25 and p220 isolated from insulin-stimulated cells shows only phosphoserine, even when extracts are prepared in the presence of 1 mM vanadate (data not shown). In addition to eIF-4F, other proteins involved in the mRNA binding step of protein synthesis initiation are phosphorylated in response to insulin. Exposure of 3T3-L1 cells to insulin promotes the phosphorylation of eIF-4B, eIF-3 p120, and ribosomal protein S6, typically with a 2.5-3-fold increase in phosphorylation (summarized in Table I). In studies with reticulocytes, purification of eIF-4B and eIF-3 have suggested that the increased phosphorylation observed reflects the total cellular pools of these factors (data not shown).

**TABLE I**

Comparison of stimulation of phosphorylation of initiation factors and S6 by insulin and PMA

| Condition          | eIF-4F | eIF-4F | eIF-4B | eIF-3 p120 or p120 | S6 |
|--------------------|--------|--------|--------|------------------|----|
| No addition        | 1.0    | 1.0    | 1.0    | 1.0              |    |
| + PMA              | 2.5-5.0| 2.5-5.0| 1.5-2.5| 2.4-4.0          | 1.5-2.0|
| 3T3-L1 cells       | 1.0    | 1.0    | 1.0    | 1.0              |    |
| + PMA              | 4.1    | 4.2    | 2.4    | 2.2              | 1.5-2.0|
| + Insulin          | 2.5    | 5.0    | 3.2    | 3.2              | 2.5-3.0|
| 3T3-L1 cells down- | 1.1    | 0.9    | 1.0    | 1.0              | 1.1 |
| regulated with PMA|        |        |        |                  |    |
| + PMA              | 1.3    | 5.3    | 2.0    | 1.6              | 3.0 |

* Data for reticulocytes taken from (15). Data for 3T3-L1 cells are averages of six separate experiments; data for down-regulated 3T3-L1 cells are an average of three separate experiments.
In all cases, mapping of labeled ~25 gives rise to a single tryptic digestion and two-dimensional phosphopeptide map-ment or phosphorylation of eIF-4F by protein kinase C in
presence of phosphoserine (data not shown). Stimulation of
~25 is fully phosphorylated during the down-regulation proc-
tion of ~25 in response to insulin is not due to the fact that
phosphorylation of any initiation factor studied or S6 can be
phosphorylated by both a PMA-dependent and insulin-de-
pendent pathway, whereas phosphorylation of eIF-4F p25 and
eIF-3 p120 is stimulated primarily upon activation of protein
kinase C. In addition, these studies suggest that in 3T3-L1
cells, insulin may increase the activity of protein kinase C,
either directly or indirectly. In light of the data presented
above, insulin also activates protease-activated kinase II,
which appears to be one of the protein kinases responsible for
the insulin-induced phosphorylation of these initiation factors
but is not the only protein kinase modifying eIF-4F p220 in
vivo. Studies are currently underway to determine how these
phosphorylation events lead to stimulation of protein synthe-
sis in these cells. Coordinate phosphorylation of eIF-4F-4F,
eIF-4B, eIF-3, and S6 could be responsible for stimulation of
protein synthesis and for altered translation of specific classes
of mRNA observed upon stimulation of quiescent cells by
hormones and growth factors (4, 17).

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