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Interleukin 3 stimulates protein synthesis by regulating double-stranded RNA-dependent protein kinase

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ABSTRACT **In a murine interleukin 3 (IL-3)-dependent cell line, IL-3 deprivation resulted in increased autophosphorylation of double-stranded RNA-dependent protein kinase (PKR) that has been reported to inhibit protein synthesis by phosphorylating the α subunit of eukaryotic initiation factor 2 (eIF-2α). Autophosphorylation was characterized by a shift up in mobility of PKR on SDS/PAGE gels from a 60- to a 64-kDa form. *In vitro* kinase studies comparing the autophosphorylated 64-kDa PKR with the nonphosphorylated 60-kDa PKR confirmed that only the 64-kDa form was active for eIF-2α phosphorylation. PKR activation *in vivo* was associated with phosphorylation of eIF-2α and inhibition of protein synthesis. Addition of IL-3 to deprived cells elicited a reciprocal response characterized by the rapid dephosphorylation of PKR and eIF-2α, indicating inactivation of PKR. This was rapidly followed by the full recovery of protein synthesis. Furthermore, upon IL-3 addition, a 97-kDa phosphotyrosine-containing protein becomes rapidly and transiently associated with PKR prior to dephosphorylation of PKR and eIF-2α. Genistein, a tyrosine kinase inhibitor, blocks both phosphorylation of the 97-kDa phosphoprotein and protein synthesis after IL-3 addition, suggesting a role for the 97-kDa phosphoprotein in the mechanism of inactivation of PKR and stimulation of protein synthesis. Thus, IL-3 appears to positively regulate protein synthesis by inducing the inactivation of PKR in a growth factor signaling pathway.**

Hematopoietic growth factors such as interleukin 3 (IL-3) stimulate growth by triggering postreceptor signaling pathways (1). The signal(s) has been shown to result in the activation of growth-related transcription factors such as myc, fos, and jun (2). However, implicit in growth factor action is the requirement for the synthesis of structural and regulatory proteins involved in growth. While a great deal is known about how the initiation and elongation factors required for protein synthesis are regulated (3–6), little is known about the molecular mechanism(s) by which growth factors might couple to and activate this process.

One of the best characterized mechanisms for regulating protein synthesis is the reversible phosphorylation of the α subunit of eukaryotic protein synthesis initiation factor 2, eIF-2α (3–6). Phosphorylation of eIF-2α increases the stability of complexes formed between eIF-2α and eIF-2B (7). Since eIF-2B is required for the exchange of GDP for GTP in the recycling of eIF-2 in the formation of the eIF-2·GTP·Met-tRNA ternary complex and exists in cells in relatively low molar quantities with respect to eIF-2, phosphorylation of only a limited amount (i.e., 20–25%) of eIF-2α is sufficient to sequester virtually all of the eIF-2B, resulting in inhibition of protein synthesis (7). There are currently three known eIF-2α-specific protein kinases (8): the interferon-inducible double-stranded (ds) RNA-dependent kinase (PKR), a heme-sensitive kinase, and the yeast GCN2, a kinase regulated by amino acid availability. All three kinases possess the ability to autophosphorylate on an as yet unidentified serine residue in an apparently intermolecular reaction that serves to activate the kinase, leading to phosphorylation of eIF-2α (8). However, any role for growth factor regulation of an eIF-2α kinase has not yet been identified.

PKR was first identified as a component of the host defense mechanism induced by interferon (9, 10). In interferon-treated cells, virus infection leads to activation of PKR by autophosphorylation, followed by eIF-2α phosphorylation and inhibition of protein synthesis (11). *In vitro*, PKR can be activated by synthetic dsRNA, such as poly(I)·poly(C), and natural dsRNA forms such as reovirus genomic RNA or domains of mRNAs that form stable double-stranded structures (11, 12). The negative control of protein synthesis by eIF-2α-specific protein kinases may serve a basic protective function in eukaryotic cells under conditions of growth stress. Such a negative regulatory mechanism might be expected to be counterbalanced by an as yet uncharacterized stimulatory pathway. Using IL-3-dependent cells, we find that IL-3 can stimulate protein synthesis apparently by decreasing the steady-state phosphorylation status of PKR and eIF-2α.

METHODS

Cell Culture. The murine IL-3-dependent NFS/N1.H7 cells (13) were maintained in RPMI 1640 medium supplemented with 20% (vol/vol) WEHI-3B cell conditioned medium as a source of IL-3. Synthetic murine IL-3 (14) was used at 1 μg/ml for the stimulation of cells. In some experiments, cells were treated with mouse interferon α + β (Lee Biomolecular Laboratories, San Diego; 500 units/ml) for 18 hr.

Preparation of Antisera Against Murine PKR. Polyclonal antibody Ab-1 was produced in a rabbit immunized with a recombinant glutathione *S*-transferase (GST) fusion protein corresponding to aa 1–229 of murine PKR, which was prepared by cloning a *Bam*HI–*Eco*RI fragment of a murine PKR cDNA (15) into pGEX-3X (Pharmacia) (16). The antibody was affinity-purified using PKR isolated from *Escherichia coli* transfected with a full-length PKR cDNA in pET11c (15). A rabbit anti-peptide polyclonal antibody, Ab-2, was raised against a synthetic peptide corresponding to aa 502–514 of the C terminus of murine PKR.

Immunoblot Analysis. Exponentially growing cells were washed three times and suspended at 1×10^7 cells per ml in RPMI 1640 medium before IL-3 treatment. Cells were lysed in buffer A [50 mM Tris·HCl, pH 7.3/150 mM NaCl/0.5% Triton X-100/1 mM EDTA/20 mM sodium fluoride/1 mM sodium chymostatin orthovanadate/1 μM okadaic acid, so-

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Abbreviations: PKR, double-stranded RNA-dependent protein kinase; eIF-2α, α subunit of eukaryotic initiation factor-2; IL-3, interleukin 3; ds, double stranded; GST, glutathione *S*-transferase.

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dium salt (LC Services, Woburn, MA)/(20 μg/ml)/bestatin (20μg/ml)/1 mM phenylmethylsulfonyl fluoride]. Total cell lysate (100 μg) or immunoprecipitated protein from the lysate was subjected to electrophoresis on an SDS/6.5% polyacrylamide gel followed by immunoblot analysis with anti-PKR antibody and ^{125}I-labeled protein A (Amersham) as described (17). To demonstrate the effects of phosphorylation on the mobility of PKR on SDS/PAGE, total cell lysate desalted with a G-25 column was incubated with 25 mM Tris·HCl, pH 8.0/5 mM $MgCl_2$, 2 μM ATP, or poly(I)·poly(C) (Pharmacia; 0.1 μg/ml) plus 2 μM ATP for 30 min at 30°C. A treated sample was further incubated with calf intestinal alkaline phosphatase (100 unit/ml) for 15 min at 30°C.

Phosphocellulose Column Chromatography. Phosphorylated and nonphosphorylated PKR were separated with phosphocellulose column chromatography based on the method as described (18). Cells were lysed in buffer B [50 mM Tris·HCl, pH 7.0/0.5% Triton X-100/1 mM EDTA/20 mM sodium fluoride/1 mM sodium orthovanadate/1 μM okadaic acid, sodium salt/chymostatin (20 μg/ml)/bestatin (20 μg/ml)/1 mM phenylmethylsulfonyl fluoride] containing 50 mM NaCl. The cell lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was loaded onto a phosphocellulose (Whatman) column equilibrated with the same buffer. The flowthrough and eluate with 2 column volumes of buffer B were collected as a low salt fraction. The column was washed with buffer B containing 100 mM NaCl, and a high salt fraction was collected with 2 column volumes of buffer B containing 500 mM NaCl.

Measurement of PKR Activity for eIF-2α Phosphorylation *in Vitro*. PKR in eluates of phosphocellulose columns was immunoprecipitated using Ab-2 conjugated with protein A-agarose beads. The immune complexes were washed twice in buffer B containing 1 M NaCl and twice in kinase buffer containing 25 mM Tris·HCl (pH 7.6), 2 mM $MgCl_2$, and 2 mM $MnCl_2$, followed by incubation in 50 μl kinase buffer containing 0.5 μg of purified rabbit reticulocyte eIF-2α (19), 20 μM ATP, 1 mM dithiothreitol, and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) for 10 min at 30°C, as described (20). The phosphorylated proteins were analyzed by SDS/PAGE (10% gel) and autoradiography.

Metabolic Labeling. Approximately 2 × 10^7 cells were incubated with [^{32}P]orthophosphoric acid (ICN) at 0.1 mCi/ml or [^{35}S]methionine (Tran^{35}S-label, ICN) at 0.5 mCi/ml in the absence of IL-3 for 2 hr prior to the addition of IL-3 (1 μg/ml). Cells were collected and lysed in buffer A, and PKR was immunoprecipitated with Ab-1. Two-dimensional phosphoamino acid analysis was performed as described (21).

Measurement of Cellular Protein Synthesis. Approximately 1 × 10^6 cells were incubated with L-[U-^{14}C]amino acid mixture (Amersham) at 5 μCi/ml for 10 min at 37°C at the indicated times. The reaction was terminated by the addition of ice-cold 20% (wt/vol) trichloroacetic acid and the radioactivity in the acid-precipitable fraction was measured in a scintillation counter.

Analysis of eIF-2α Phosphorylation. Cells were lysed in 10 mM Hepes, pH 7.2/5 mM EDTA/150 mM KCl/0.05% SDS/1% Triton X-100/20 mM sodium fluoride/20 mM sodium pyrophosphate/20 mM β-glycerophosphate/20 mM sodium molybdate/aprotinin (100 μg/ml)/leupeptin (50 μg/ml)/1 mM dithiothreitol. The acetone-precipitated extract was solubilized in sample buffer and 1 × 10^6 cell equivalents of protein was subjected to vertical slab gel isoelectric focusing and immunoblot analysis with anti-eIF-2α antibody, as described (22).

RESULTS AND DISCUSSION

NFS/N1.H7 Cells Express Phosphorylated and Nonphosphorylated Forms of PKR. To investigate the function of PKR in IL-3 signaling, we raised PKR antiserum (Ab-1) by immunizing a rabbit with a recombinant PKR fusion protein. Immunoblot analysis shows that Ab-1 recognizes a 60- to 64-kDa protein expressed in murine IL-3-dependent NFS/N1.H7 cells (Fig. 1*A*). The intensity of the specifically recognized band is increased in whole-cell lysates or after immunoprecipitation with Ab-1 from cells treated with interferon for 18 hr. This demonstrates the well-described interferon-inducible nature of PKR (10, 11).

Although the predicted molecular mass of murine PKR from the amino acid sequence is 58 kDa (16), PKR actually migrates as a diffuse 60- to 64-kDa band. As reported (23–25), this migration pattern likely results from the differential phosphorylation of PKR. The 60-kDa form of PKR predominates in growing cells (Fig. 1*A*). Incubation of a cell extract with ATP and poly(I)·poly(C), a synthetic dsRNA known to induce autophosphorylation of PKR (10, 23, 24), caused a complete shift from the 60-kDa to the 64-kDa form compared

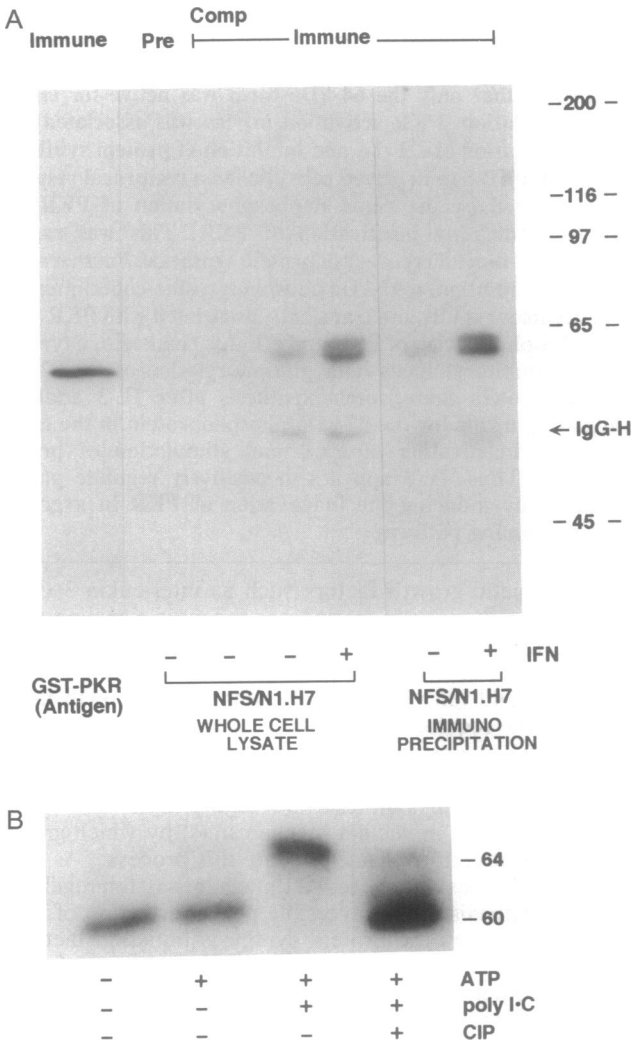

FIG. 1. Immunoblot analysis of PKR in murine IL-3-dependent NFS/N1.H7 cells. (*A*) Cells were incubated with (+) or without (−) murine interferon (IFN) for 18 hr and 100 μg of whole cell lysate protein was loaded either directly or after immunoprecipitation with Ab-1 as indicated. The GST–PKR (aa 1–229) used for the preparation of Ab-1 is shown as a positive control. Lanes: Immune, immune serum; Pre, preimmune serum; Comp, competition using immune serum preabsorbed with the GST–PKR antigen. (*B*) Cell lysate was incubated with (+) or without (−) ATP in the presence (+) or absence (−) of poly(I)·poly(C). A sample treated with ATP and poly(I)·poly(C) (poly I·C) was further incubated with calf intestinal alkaline phosphatase (CIP).

with either the untreated sample or the sample treated with ATP alone (Fig. 1*B*). These data suggest that the 64-kDa form is the autophosphorylated form. To confirm this, the sample treated with ATP and poly(I)·poly(C) was incubated with calf intestinal alkaline phosphatase (Fig. 1*B*). This resulted in a shift from the 64-kDa to the 60-kDa form, confirming the 60-kDa form as nonphosphorylated PKR.

IL-3 Regulates the Rate of Protein Synthesis and the Phosphorylation Status of PKR and eIF-2α. When growing cells are deprived of IL-3 for 120 min under serum-free conditions, there is a steady decrease in the rate of total protein synthesis to 60% of that for nondeprived growing cells (Fig. 2*A*). During the starvation period, there is a concomitant increase in the proportion of phosphorylated to unphosphorylated PKR as measured by an increase in the ratio of the 64- to the 60-kDa form (Fig. 2 *A* and *B*). The maximum extent of PKR phosphorylation at 120 min is estimated by densitometry to be ≈20%. Furthermore, a concomitant increase occurs in the phosphorylation of eIF-2α (3–8), the physiological substrate of activated PKR (Fig. 2*C*). This is temporally correlated with increased PKR phosphorylation and activation and decreased protein synthesis (Fig. 2 *A* and *B*). The increased ratio of phosphorylated to nonphosphorylated eIF-2α observed indicates that during IL-3 deprivation up to 20% of the total cellular pool of eIF-2α becomes phosphorylated (Fig. 2 *A* and *C*), an amount previously demonstrated to be sufficient to virtually shut down protein synthesis in a reticulocyte translation system (7).

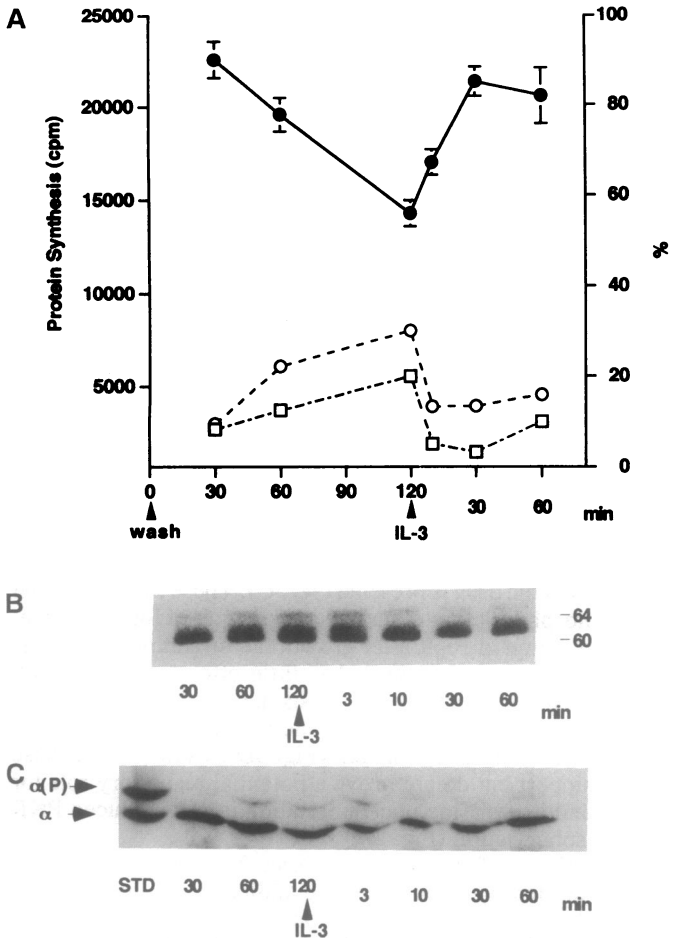

FIG. 2. Effect of IL-3 on the protein synthesis rate and phosphorylation of PKR and eIF-2α. After depriving cells of IL-3 for 120 min, IL-3 (1 μg/ml) was added. At the indicated times, the protein synthesis rate was measured (*A*) and the phosphorylation status of PKR (*B*) and eIF-2α (*C*) was determined by immunoblot analysis. The protein synthesis assay was performed six times and the mean ± SEM is indicated (●). The percent of phosphorylated (64 kDa) to unphosphorylated (60 kDa) PKR (□) and phosphorylated eIF-2α [α(P)] to unphosphorylated eIF-2α (α) (○) was determined by densitometric analysis of data in *B* and *C*. Purified phosphorylated and unphosphorylated rabbit reticulocyte eIF-2α is in lane STD.

While the inhibition of protein synthesis is rapid, it can be completely reversed within 30 min by IL-3 addition (Fig. 2*A*). This dramatic reversal is temporally associated with a reduction in the phosphorylation state of both PKR and eIF-2α, strongly suggesting that PKR is inactivated after IL-3 addition (Fig. 2 *B* and *C*).

It is well known that autophosphorylation of PKR *in vitro* coincides with increased enzymatic activity resulting in phosphorylation of eIF-2α (9–11, 20). Therefore, we tested whether the phosphorylated 64-kDa form of PKR, which accumulates after IL-3 deprivation *in vivo*, but not the nonphosphorylated 60-kDa form, was enzymatically active. The 64- and 60-kDa forms of PKR present in IL-3-deprived cells were first separated by phosphocellulose chromatography. The 64-kDa phosphorylated form of PKR failed to bind to phosphocellulose under low salt conditions, whereas the 60-kDa nonphosphorylated form of PKR could bind and was eluted under high salt conditions (Fig. 3*A*). To perform the *in vitro* kinase assay, a second PKR antibody (Ab-2) was raised against another murine PKR peptide since initial studies indicated that binding of Ab-1 to PKR blocked PKR's ability to phosphorylate eIF-2α (data not shown), presumably by inhibiting substrate binding. Quantitative immunoblot analysis of the separated 64- and 60-kDa PKR forms was used to estimate and adjust the PKR protein content of each kinase assay (Fig. 3*B*). Results demonstrate that eIF-2α was phosphorylated by the 64- but not the 60-kDa form of PKR. No eIF-2α kinase activity was detected in the immunoprecipitates with the preimmune serum demonstrating both antibody specificity and a lack of intrinsic kinase activity in the purified preparation of eIF-2α. Of note, the same apparent amount of [^{32}P]phosphate was incorporated into either the 64-kDa or 60-kDa PKR. However, the total amount of phosphate is much higher for the 64-kDa form, which was already heavily phosphorylated *in vivo* and would be unable to incorporate much additional phosphate *in vitro*. Since the degree of visual ^{32}P-labeling of both PKR forms is similar, this indicates that only a very small amount of [^{32}P]phosphate is actually incorporated into either form *in vitro*. This finding is supported by the lack of eIF-2α kinase activity observed in the 60-kDa form of PKR (Fig. 3*B*). Moreover, since no additional activator such as dsRNA is present in the assay mixture, the autophosphorylation observed on the 60-kDa PKR probably does not occur at the site(s) necessary for enzymatic activation. These results indicate that the 64-kDa form isolated after IL-3 deprivation is indeed the active form.

A 97-kDa Phosphotyrosine-Containing Protein Transiently Associates with PKR After IL-3 Addition. Dephosphorylation of PKR upon IL-3 addition was confirmed by metabolic labeling studies. Cells were incubated with [^{32}P]orthophosphoric acid in the absence of IL-3 for 120 min, followed by the addition of IL-3 for 10 min (Fig. 4*A*). These results demonstrate that a rapid reduction in the total phosphorylation of PKR occurs since the total protein level of PKR does not change during IL-3 treatment (Fig. 2*B*). While the mechanism by which IL-3 might trigger PKR dephosphorylation remains unclear, it could result from the stimulation of a phosphatase (26) or, conversely, from the inhibition of the ability of PKR to autophosphorylate. Interestingly, IL-3-mediated dephosphorylation of PKR is accompanied by the coimmunoprecipitation of a 97-kDa phosphoprotein (pp97) (Fig. 4*A*). Since Ab-1 does not recognize pp97 on an immunoblot, this appears to represent a unique protein interaction triggered by IL-3. The rapid association of PKR with pp97 suggests that such an interaction might be involved in regulating the phosphoryla-

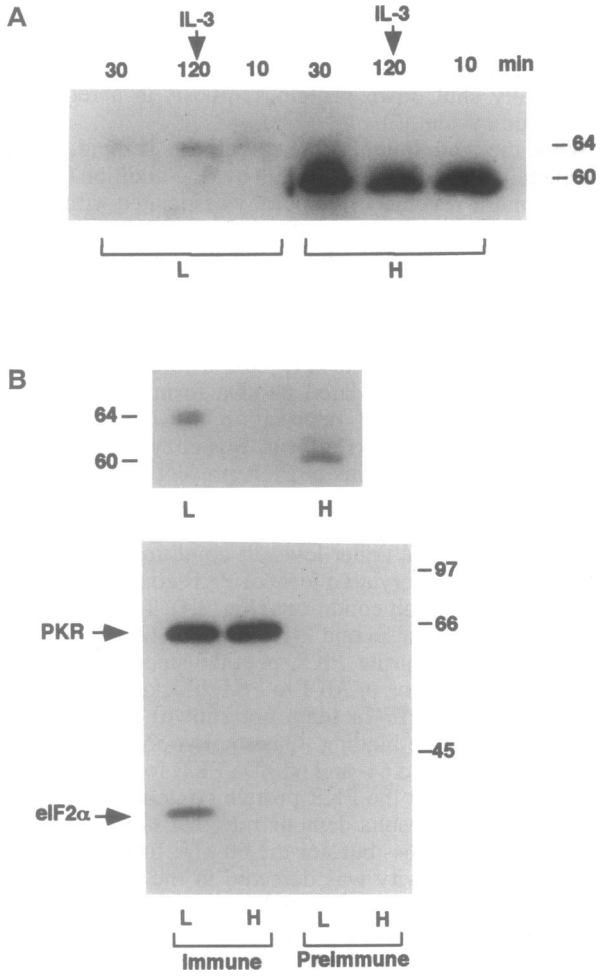

FIG. 3. IL-3 induces dephosphorylation and inactivation of PKR. (*A*) After cells were deprived of IL-3 for 120 min, IL-3 was added as in Fig. 2. At the indicated times, cells lysates were loaded onto phosphocellulose columns. The low salt (lanes L; 50 mM NaCl) and the high salt (lanes H; 500 mM NaCl) eluates from the column were analyzed on an immunoblot. (*B*) Equal amounts of PKR protein in the L and H fractions were immunoprecipitated with Ab-2. The PKR from each fraction was then incubated with [γ-^{32}P]ATP in the presence of rabbit reticulocyte eIF-2α as a substrate. Preimmune serum was used as the negative control for the immunoprecipitation.

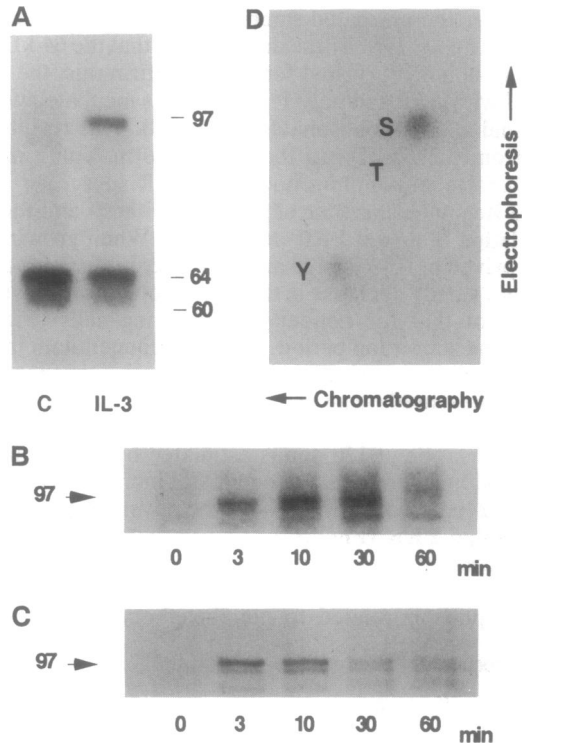

FIG. 4. IL-3 mediates the coimmunoprecipitation of pp97 with PKR. (*A*) Cells were labeled with [^{32}P]orthophosphoric acid in the absence of IL-3 for 120 min and then incubated with (lane IL-3) or without (lane C) IL-3 for 10 min. PKR was immunoprecipitated from the cell lysates with Ab-1. The time-dependent association of pp97 with PKR after incubation with IL-3 for the indicated times is demonstrated using cells labeled with either [^{32}P]orthophosphoric acid (*B*) or [^{35}S]methionine (*C*). (*D*) Phosphoamino acid analysis of pp97.

tion state of PKR. To evaluate this possibility, the kinetics of interaction between PKR and pp97 were studied by metabolic labeling of cells with both [^{32}P]orthophosphoric acid (Fig. 4*B*) and [^{35}S]methionine (Fig. 4*C*). The results demonstrate that the association is rapid, occurring by 3 min, and clearly precedes PKR dephosphorylation. Furthermore, IL-3 treatment induces the transient and reversible association of PKR and pp97 since both ^{32}P- and ^{35}S-labeled pp97 decrease with time after IL-3 addition. This finding is consistent with a potential regulatory role for pp97.

Genistein Inhibits IL-3-Stimulated Phosphorylation of pp97 and the Dephosphorylation of PKR and Protein Synthesis. A number of studies have suggested the necessary involvement of protein tyrosine kinases in the cytokine signaling process (1). Interestingly, a phosphoamino acid analysis reveals that pp97 is phosphorylated on tyrosine and serine residues (Fig. 4*D*). While the function, if any, of pp97 remains unknown, its tyrosine phosphorylation may be essential. Thus when genistein, a selective inhibitor of protein tyrosine kinase (27), was added to cells prior to IL-3 the coimmunoprecipitation of a ^{32}P-labeled pp97 with PKR is apparently prevented (Fig. 5*A*). This blockade actually results from the failure of pp97 to become/remain phosphorylated after genistein treatment since pp97 can still be detected in PKR immunoprecipitates from cells labeled with [^{35}S]methionine and treated with IL-3 (Fig. 5*B*). This indicates that tyrosine phosphorylation of pp97 is apparently not required for the physical association with PKR. However, in the presence of genistein, the IL-3 triggered reduction in PKR phosphorylation is clearly blocked since immunoblot analysis shows that the 64-kDa form decreases by 70% in the absence of genistein but only 15% when the drug is present (Fig. 5*C*). These results suggest that phosphorylation of pp97 may be required for any potential regulation of PKR after growth factor addition. Furthermore, while genistein itself has little effect on the already decreased rate of protein synthesis in cells deprived of IL-3, it dramatically blocks the rapid increase in protein synthesis observed when IL-3 is added (Fig. 5*D*). Collectively, these data support but do not prove a potential regulatory role for pp97 and its tyrosine phosphorylation in IL-3-induced PKR dephosphorylation (Fig. 2).

Stimulation of protein synthesis has been reported to occur in serum-deprived cells after the addition of dialyzed serum (28, 29). This occurs in association with dephosphorylation of eIF-2α although the mechanism involved and the identification of an eIF-2α protein kinase(s) has not previously been addressed. We demonstrate here that PKR occupies a central regulatory role in protein synthesis stimulated by a growth factor, IL-3. Our results may explain how serum, which contains growth factors, can stimulate protein synthesis in close association with dephosphorylation of eIF-2α. Of particular note, PKR has recently been found to have potential tumor suppressor activity as indicated by the inactive kinase mutant forms of PKR acting in a dominant-negative fashion to permit both transformation of NIH 3T3 cells and tumor-

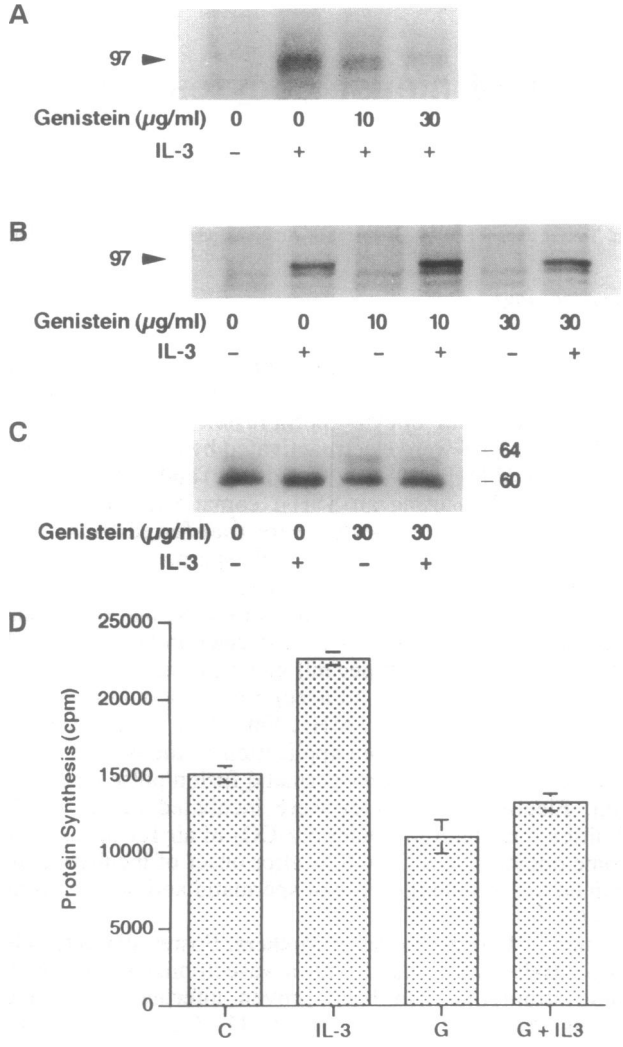

FIG. 5. Effect of genistein on the phosphorylation of pp97, PKR, and protein synthesis. pp97 was coimmunoprecipitated using Ab-1 from cells labeled with [^{32}P]orthophosphoric acid (*A*) or [^{35}S]methionine (*B*). Cells were first incubated in the presence or absence of genistein at the indicated concentrations for 120 min prior to the addition of IL-3 for 10 min. (*C*) Immunoblot analysis of PKR from cells treated with (+) or without (−) IL-3 in the presence or absence of genistein (30 μg/ml). (*D*) Protein synthesis rates were measured in cells deprived of IL-3 for 120 min in the presence (bar G) or absence (bar C) of genistein (30 μg/ml) followed by the addition of IL-3 (IL-3) for 10 min. The experiment was performed three times and the error bars represent the mean ± SEM.

igenic growth of such cells when injected into nude mice (30–32). In addition, oncogenic *ras* apparently can induce an inhibitor of PKR in BALB/c 3T3 cells that blocks platelet-derived growth factor- or interferon-mediated signaling (33). These reports point to a positive regulatory role for PKR during cell growth, which contrasts with its well demonstrated negative role in viral-induced host defense mechanisms characterized by the inhibition of protein synthesis (10, 11). Collectively, such findings, when coupled with those reported here, strongly support a central role for PKR regulation in a growth factor-stimulated pathway resulting in protein synthesis.

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