Gab-1-mediated IGF-1 Signaling in IRS-1-deficient 3T3 Fibroblasts*

(Received for publication, August 16, 1999, and in revised form, December 9, 1999)

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The insulin receptor substrate (IRS) family of proteins mediate a variety of intracellular signaling events by serving as signaling platforms downstream of several receptor tyrosine kinases including the insulin and insulin-like growth factor-1 (IGF-1) receptors. Recently, several new members of this family have been identified including IRS-3, IRS-4, and growth factor receptor-binding protein 2-associated binder-1 (Gab-1). 3T3 cell lines derived from IRS-1-deficient embryos exhibit a 70–80% reduction in IGF-1-stimulated S-phase entry and a parallel decrease in the induction of the immediate-early genes c-fos and egr-1 but unaltered activation of the mitogen-activated protein kinases extracellular signal-regulated kinase-1 and extracellular signal-regulated kinase-2. Reconstitution of IRS-1 expression in IRS-1-deficient fibroblasts by retroviral mediated gene transduction is capable of restoring these defects. Overexpression of Gab-1 in IRS-1-deficient fibroblasts also results in the restoration of egr-1 induction to levels similar to those achieved by IRS-1 reconstitution and markedly increases IGF-1-stimulated S-phase progression. Gab-1 is capable of regulating these biological end points despite the absence of IGF-1 stimulated tyrosine phosphorylation. These data provide evidence that Gab-1 may serve as a unique signaling intermediate in insulin/IGF-1 signaling for induction of early gene expression and stimulation of mitogenesis without direct tyrosine phosphorylation.

Insulin and insulin-like growth factor 1 (IGF-1)1 elicit diverse biological effects by binding to and activating their cognate tyrosine kinase receptors (1, 2). The major substrates for both the insulin and IGF-1 receptors are the closely related high molecular weight proteins insulin receptor substrates-1 and -2 (IRS-1 and -2), which become rapidly phosphorylated on multiple tyrosine residues in response to ligand stimulation (3–5). These phosphorylated substrates then bind adapter proteins containing Src homology 2 (SH2) domains, such as the regulatory subunits of PI 3-kinase (6), protein-tyrosine phosphatase SH2 domain containing phosphatase-2/Syp (7), and the growth factor receptor-binding protein 2 (Grb2), which links signaling via SOS to activation of the Ras complex (8, 9). In addition, depending on the cell type, the activated insulin and IGF-1 receptors phosphorylate several other substrates, including IRS-3 (10–13), IRS-4 (14), several isoforms of Src homologous and collagen (15–17), and grb-2-associated binder-1 (Gab-1) (18). Together, these intermediate signals stimulate a variety of downstream biological pathways, such as cytochrome synthesis, glucose transport, gene expression, and mitogenesis.

Gab-1 was initially identified in a cDNA library of glioblastoma tumors based upon its ability to bind Grb-2 (18). Gab-1 shares functional and structural homology with the IRS family of proteins, including an N-terminal pleckstrin homology (PH) domain, several tyrosine phosphorylation sites, and two potential SH3 binding proline-rich regions. Several studies have also demonstrated that Gab-1 functions as a substrate and docking protein downstream in the signaling pathways of several receptor tyrosine kinases, including the receptors for epidermal growth factor (EGF), insulin, fibroblast growth factor, nerve growth factor, hepatocyte growth factor, and various cytokines such as interleukin-3, interleukin-6, interferon-α, and interferon-γ (18–21). Like the IRS proteins, tyrosine-phosphorylated Gab-1 has been shown to interact with several Src homologous and collagen-containing adapter molecules, such as the p85 regulatory subunit of PI 3-kinase, SH2 domain containing phosphatase-2, and Grb-2 (18, 21, 22). In addition, Gab-1 can interact with phospholipase C-γ, which does not interact with IRS-1.

Previous studies in our laboratory have demonstrated a specific role for IRS-1 in insulin and IGF-1-mediated signaling (23). Thus, although there is a high degree of homology and structural similarity among the IRS family members, cells with a homozgyous deficiency of IRS-1 show a reduced mitogenic response that cannot be recovered by the overexpression of IRS-2 (23). These data highlight the potential for specific IRS proteins to uniquely mediate distinct downstream biological events. In the present study, we have investigated the role of Gab-1 in mediating IGF-1 signaling by overexpressing Gab-1 in IRS-1-deficient 3T3 fibroblasts by retroviral mediated gene transfer. Our studies revealed that Gab-1 overexpression produces a unique pattern of downstream signals in IRS-1-deficient fibroblasts, despite an absence of detectable IGF-1-stimulated tyrosine phosphorylation, suggesting a novel role and mechanism for this substrate in insulin/IGF-1 action.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant IGF-1 was purchased from Peprotech Inc.(Rocky Hill, NJ). The Gab-1 cDNA was a generous gift of Albert Wong (Kimmel Cancer Institute, Philadelphia, PA). Polyclonal antibodies to Gab-1 and IRS-2 and monoclonal antibody to phosphotyrosine were a generous gift from Morris F. White (Joslin Diabetes Center, Boston, MA). Antibodies to MAP kinase and IRS-1 were prepared as...
Inhibitor (Sigma), and 5 mM HCl (pH 7.5), 137 mM NaCl, 0.05% Tween-20, 3% bovine serum albumin was then resolved directly in SDS-polyacrylamide gels after boiling in SDS sample buffer for 3 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. After transfer to nylon membranes, blots were hybridized with the indicated probe. A probe specific for IRS-1 was generated by the directional cloning of an EcoRI/SalI fragment corresponding to the C-terminal 1010 base pairs of murine IRS-1 into pBluescript. A probe specific for IRS-2 was generated by polymerase chain reaction amplification of the C-terminal 970 base pair region (5100–5070) of murine IRS-2 and subcloned into the EcoRI/SalI sites of the pCR2.1-TOPO vector. A Gab-1-specific probe corresponding to the first 1679 base pairs of human Gab-1 was constructed by Xho1 digestion of pBSGab-1 and subsequent vector religation. Egr-1 expression was assessed using a full-length cDNA to the human egr-1 gene. All probes used for Northern blot analysis were labeled using a random primed labeling kit (Amersham Pharmacia Biotech). After an overnight incubation at 42°C, blots were washed three times in 1× SSC (1× SSC is 0.15 mM NaCl plus 0.015 mM sodium citrate), 0.1% SDS for 20 min each time at room temperature and for 30 min in 0.1× SSC, 0.1% SDS at 52°C. Filters were air dried and subjected to autoradiography.

RESULTS

Overexpression of Gab-1 and Reconstitution of IRS-1—IRS-1-deficient 3T3 cells were established from embryonic fibroblasts derived from IRS-1-deficient mice (23). As described previously, these cells have a reduced response to IGF-1-stimulated mitogenesis and early gene expression (23). To investigate the potential role of Gab-1 in IGF-1-mediated signaling, we overexpressed Gab-1 (KO+ Gab-1) or IRS-1 (KO− IRS-1) in these IRS-1−/− 3T3 cell lines by retroviral mediated gene transduction. By Northern blot analysis, no IRS-1 message could be detected in IRS-1+ Gab-1 or IRS-1+ KO+ Gab-1 cells using a probe specific for IRS-1, whereas in KO cells reconstituted with IRS-1 using retroviral mediated gene transfer, a transcript of 9.0 kilobases corresponding to the human IRS-1 mRNA could be easily detected (Fig. 1A). Northern blot analysis using an IRS-2-specific probe indicated that all cell lines expressed equivalent levels of IRS-2 message. On parallel blots of total RNA, no Gab-1 message was detected in IRS-1−/− cells either before or after IRS-1 expression using a probe specific for human Gab-1. By contrast, a 4.2-kilobase transcript corresponding to Gab-1 mRNA was easily detected in KO cells transfected with Gab-1.

The level of protein expression of IRS-1, IRS-2, and Gab-1 was assessed by Western blot analysis on cell lysates of the same cells. Consistent with Northern blot analysis, immunoblotting using an antibody specific to the C-terminal region of IRS-1 confirmed the absence of IRS-1 protein in KO and KO+ Gab-1 cells as well as the reconstitution of IRS-1 in KO− IRS-1 cell lines (Fig. 1B). By contrast, all cell lines expressed equivalent levels of IRS-2 protein, as determined by Western blot analysis with anti-IRS-2 antibodies. Immunoblots probed with an antibody raised against the C terminus of Gab-1 indicated the presence of low endogenous expression of Gab-1 in KO and KO− IRS-1 cells and a 3.4-fold overexpression of human Gab-1 when compared with KO and KO+ IRS-1 cell lines. These data confirm the reconstitution of IRS-1 and the overexpression of Gab-1 in KO+ IRS-1 and KO− Gab-1 cell lines, respectively, and indicate that no alterations in the level of IRS-2

In Vitro Kinase Reactions—Two hundred micrograms of cellular protein was subjected to immunoprecipitation with a polyclonal antiserum to ERK-1 and ERK-2, and immunocomplexes were collected with protein A-Sepharose beads. After two washes in lysis buffer and two washes in 50 mM HEPES, 10 mM MgCl2 (pH 7.4), beads were resuspended in 30 μl of reaction buffer, and the reactions were started by the addition of 5 μCi of [γ-32P]ATP, 100 μM ATP, 1 μg of protein A-Sepharose (Amersham Pharmacia Biotech) in phosphate-buffered saline for 1 h at 4°C. After two washes in buffer A containing 0.1% SDS, protein complexes were liberated from beads by boiling in SDS sample buffer for 3 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Immunodetection was performed after the membranes were blocked for 2 h at room temperature in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.05% Tween-20, 3% bovine serum albumin followed by incubation with the appropriate antibody at the indicated concentration for 2 h at room temperature. Specifically bound antibodies were detected with 125I-labeled protein A and autoradiography.

In Vivo Kinase Reactions—Two hundred micrograms of cellular protein was subjected to immunoprecipitation with a polyclonal antiserum to ERK-1 and ERK-2, and immunocomplexes were collected with protein A-Sepharose beads. After two washes in lysis buffer and two washes in 50 mM HEPES, 10 mM MgCl2 (pH 7.4), beads were resuspended in 30 μl of reaction buffer, and the reactions were started by the addition of 5 μCi of [γ-32P]ATP, 100 μM ATP, 1 μg of protein A-Sepharose (Amersham Pharmacia Biotech) in phosphate-buffered saline for 1 h at 4°C. After two washes in buffer A containing 0.1% SDS, protein complexes were liberated from beads by boiling in SDS sample buffer for 3 min. Proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Immunodetection was performed after the membranes were blocked for 2 h at room temperature in 20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 0.05% Tween-20, 3% bovine serum albumin followed by incubation with the appropriate antibody at the indicated concentration for 2 h at room temperature. Specifically bound antibodies were detected with 125I-labeled protein A and autoradiography.
protein expression resulted from the reconstitution of IRS-1 or overexpression of Gab-1.

Absence of IGF-1-stimulated Gab-1 Tyrosine Phosphorylation—To determine whether Gab-1 is capable of becoming phosphorylated on tyrosine residues in response to IGF-1 stimulation, whole cell lysates from IRS-1 and EGF-stimulated KO; KO-IRS-1 and KO+Gab-1 cell lines were subjected to immunoprecipitation using anti-phosphotyrosine antibody and immunoblotting with anti-Gab-1 antibodies. In KO+Gab-1 cells, some basal tyrosine phosphorylation of Gab-1 could be observed (Fig. 2A). Stimulation with EGF rapidly and markedly increased the level of Gab-1 phosphorylation (18, 19). In addition, this phosphorylation resulted in decreased mobility of the protein on SDS-PAGE. In contrast, the level of tyrosine phosphorylation was not augmented in response to IGF-1 stimulation, and no mobility shift of the protein was observed. Similar results were obtained when whole cell lysates prepared from IGF-1-stimulated cell lines were subjected to immunoprecipitation with anti-Gab-1 antibody followed by immunoblotting with anti-phosphotyrosine (data not shown). Consistent with these findings, IGF-1 stimulation failed to increase the association of Gab-1 with SH2 domain containing adapter proteins such as Grb-2 and the p85 regulatory subunit of PT 3-kinase (data not shown).

To determine the level of IGF-1-stimulated IRS-1 and IRS-2 tyrosine phosphorylation and to confirm the absence of tyrosine-phosphorylated Gab-1 in response to IGF-1, whole cell lysates from IGF-1-stimulated KO; KO-IRS-1 and KO+Gab-1 cells were subjected to immunoprecipitation and immunoblotting using anti-phosphotyrosine antibody. As previously observed, KO cells lack IRS-1 tyrosine phosphorylation and the only high molecular weight phosphoprotein detected corresponded to IRS-2, as indicated by its relatively retarded mobility on SDS-gels. In KO-IRS-1 cells, IGF-1 stimulation resulted in a marked increase in IRS-1 tyrosine phosphorylation and lower levels of IRS-2 tyrosine phosphorylation. In KO+Gab-1 cells, IGF-1 stimulation resulted in the tyrosine phosphorylation of IRS-2 comparable to that seen in KO cells, but no tyrosine phosphorylated proteins were detected in the appropriate molecular weight range of Gab-1 (Fig. 2B). Thus, although Gab-1 is capable of undergoing tyrosine phosphorylation in response to EGF stimulation in 3T3 cells, it does not undergo tyrosine phosphorylation in response to IGF-1 stimulation. In addition, these data indicate that the overexpression of Gab-1 does not alter the extent or ability of IRS-2 to become tyrosine phosphorylated in response to IGF-1 stimulation, i.e. it does not appear to be a competitive substrate for the IGF-1 receptor.

MAP Kinase Activation Is Unaltered in KO+Gab-1 Cells—An important pathway activated by IGF-1 stimulation and involved in gene expression and the mitogenic response is the activation of the MAP kinase signaling cascade. Upon receptor activation, phosphorylated IRS-1, IRS-2, and SHC bind to the SH2 domain of the adapter molecular Grb-2, which in turn binds the guanine nucleotide exchange factor SOS, allowing for activation of Ras and initiation of a signaling cascade of serine/threonine kinases, including Raf, mitogen-activated protein kinase kinase, ERK-1, and ERK-2. To assess the potential impact of Gab-1 in mediating signaling via this pathway, we performed in vitro kinase assays using anti-MAP kinase immunoprecipitates and myelin basic protein as a substrate. As can be seen in Fig. 3, the MAP kinases ERK-1 and ERK-2 were activated to the same extent and also followed the same time course in response to IGF-1 in all cell lines. These data indicate that both Gab-1 and IRS-1, although capable of binding Grb-2 and activating the MAP kinases, had no apparent effect on the level and time course of IGF-1-stimulated activation of ERK-1 and ERK-2.

Immediate-Early Gene Expression Deficit in KO Cells Is Restored by Gab-1—Mice made IRS-1 deficient by targeted gene knockout exhibit intrauterine and postnatal growth retardation because of resistance to the actions of IGF-1 and insulin. In culture, IRS-1-deficient 3T3 cells also exhibit a decrease in IGF-1-mediated egr-1 and c-fos expression (23). To assess the...
At the higher concentrations of IGF-1 tested, KO S-phase progression at every point in the dose-response curve. Overexpression of Gab-1 markedly increased IGF-1-stimulated stored the potential to enter S phase (Fig. 5). Surprisingly, decreased by 70% in KO cells, and reconstitution of IRS-1 re-

Potential role for Gab-1 in mediating immediate-early gene expression, IGF-1-stimulated expression of the immediate-early gene egr-1 was assessed by Northern blot analysis. As previously reported, KO cells showed a markedly reduced expression of egr-1 in response to IGF-1 stimulation over a 60-min time course (Fig. 4A). This deficit in egr-1 expression could be restored by the reconstitution of IRS-1 in KO IRS-1 cells. Quantification of three independent experiments revealed a 50–60% reduction of egr-1 induction in KO cells when compared with KO IRS-1 cells. Interestingly, Gab-1 overexpression was also capable of restoring the defects in egr-1 expression observed in KO cells to levels 80–90% of that of KO IRS-1 cells. These data indicate the potential existence of a common pathway(s) mediated by both IRS-1 and Gab-1, which is necessary for IGF-1-stimulated activation of the immediate-early gene egr-1 (23).

Expression of Exogenous Gab-1 Restores S-Phase Entry in IRS-1-deficient Cells—Finally, to address whether overexpression of Gab-1 was capable of restoring IGF-1-stimulated cell cycle progression, we determined the level of IGF-1-stimulated incorporation of [methyl-3H]thymidine into DNA in KO IRS-1 cells. As previously observed, [methyl-3H]thymidine labeling in response to IGF-1 was decreased by 70% in KO cells, and reconstitution of IRS-1 restored the potential to enter S phase (Fig. 5). Surprisingly, overexpression of Gab-1 markedly increased IGF-1-stimulated S-phase progression at every point in the dose-response curve. At the higher concentrations of IGF-1 tested, KO Gab-1 cells exhibited a 4.2-fold higher incorporation of [methyl-3H]thymidine labeling as compared with KO IRS-1 cells (Fig. 5) and by 3-fold when compared with results obtained from wild type 3T3 fibroblast cell lines (data not shown). Thus, Gab-1 is uniquely capable of potently stimulating cell cycle progression.

**DISCUSSION**

A variety of studies have demonstrated that Gab-1 acts downstream of several receptor protein-tyrosine kinases, including the insulin and EGF receptors, by binding SH2 domain-containing signaling molecules following receptor-medi-

**FIG. 3.** IGF-1-stimulated activation of the MAP kinases. Cell extracts from KO (■), KO IRS-1 (●), and KO Gab-1 (▲) cell lines were prepared after stimulation of serum-deprived cells with 10 nm of IGF-1 for the indicated times (minutes). MAP kinase activity was then assayed on immunoprecipitates using myelin basic protein as a substrate as described under “Experimental Procedures.” After SDS-PAGE in 12% gels and electroblotting onto polyvinylidene difluoride membranes, filters were exposed to film. Quantification reflects four independent experiments. Results are expressed as percentages of maximum stimulation in KO IRS-1 cells.

**FIG. 4.** IGF-1-stimulated induction of egr-1 is restored by the overexpression of Gab-1. A, Northern blot analysis of RNA extracted from KO (●), KO IRS-1 (●), and KO Gab-1 (▲) cell lines stimulated with 10 nm of IGF-1 for the indicated times (minutes). Blots were hybridized with a probe to the full-length cDNA of egr-1 as described under “Experimental Procedures.” The upper panel shows the autoradiograph of a typical experiment. The lower panel shows the ethidium bromide-stained agarose gel, confirming integrity of the RNA and equal loading. B, quantification of the results from three independent experiments. Data are expressed as mean percentages of maximum stimulation in KO IRS-1 cells (± standard error of the means).

**FIG. 5.** Overexpression of Gab-1 causes an enhanced mitogenic response to IGF-1. IGF-1 stimulation of [3H]thymidine incorporation into DNA. Quiescent KO, KO IRS-1, and KO Gab-1 cells were stimulated with various concentrations of IGF-1 for 14 h at 37 °C. Incorporation of [3H]thymidine into DNA was assessed as described under “Experimental Procedures.” Determinations were done in triplicate, and the data are means ± standard errors of the means for four separate experiments. Results are expressed as percentages of maximum stimulation in KO IRS-1 cells.
activation of PI 3-kinase and the phosphorylation of p70s6 kinase. These studies have also demonstrated that this domain functions in insulin receptor-IRS protein recognition is unclear; the data suggest that the PH domain of IRS proteins may mediate protein-protein interactions or bind phospholipids in the membrane, which may target IRS proteins to the plasma membrane and coordinate receptor interaction (27–29). Although the PH domain of Gab-1 appears to function homologously to the PH domains of other IRS proteins based upon its ability to couple chimeric IRS-1 phosphorylation to the insulin receptor, in the context of Gab-1 the PH domain alone does not appear to be sufficient to mediate recognition by the insulin and IGF-1 receptors (20, 30–32). This hypothesis is supported by recent data that suggest that the PH and PTB domains of IRS proteins function cooperatively to mediate receptor recognition (33). One of the first steps in insulin signaling involves the autoactivation of the receptor by phosphorylation of tyrosines 1146, 1150, and 1151 in its catalytic cleft, and phosphorylation of tyrosine 960 in its juxtamembrane region (34, 35). The latter phosphorylation leads to recruitment of IRS-1 to the activated receptor and the interaction of the PTB domain of IRS-1 with phosphorylated NPXY motifs in the juxtamembrane region of the receptor. The IGF-1 receptor contains an analogous NPXY motif in its intracellular subunits, suggesting that PTB domain interaction at least partially mediates the association with this receptor. The lack of tyrosine phosphorylation of Gab-1 in response to IGF-1 stimulation in the current study suggests that the absence of a conventional PTB domain in Gab-1 may not allow for its recognition by the IGF-1 receptor. This hypothesis is supported by the failure of the yeast two-hybrid system to detect the interaction of Gab-1 with the insulin receptor (36). Although Gab-1 lacks a conventional PTB domain, Gab-1 may potentially interact with receptor tyrosine kinases via two other mechanisms. Recently, a proline-rich interaction domain in Gab-1 has been identified that mediates the interaction with the e-Met receptor (36). In addition, because the SH3 domains of Grb-2 can bind Gab-1, Grb-2 may also serve to recruit Gab-1 to the activated receptor (37, 38).

Despite the lack of apparent tyrosine phosphorylation, Gab-1 can participate in downstream signaling from the IGF-1 receptor. Immediate-early gene products often encode transcription factors that have been implicated in cellular growth and differentiation. Waters et al. (40) have shown that expression of IGF-1 antisense RNA causes a reduction of insulin-stimulated activity of a luciferase gene under the control of the c-fos serum response element, the same element that has been implicated in conferring insulin sensitivity to the c-fos promoter (39, 40). Our previous data in the IRS-1-deficient fibroblasts also provide evidence that expression of the c-fos and egr-1 genes depend on IRS-1 (23). In addition, the restoration of egr-1 expression in Gab-1 overexpressing cells indicates that Gab-1 is capable of regulating immediate-early gene expression. These data are of even greater interest considering that the IGF-1-stimulated activation of the MAP kinases is normal in IRS-1-deficient and Gab-1-overexpressing cells. Our data provide evidence for an IRS-1- or Gab-1-dependent pathway activating the egr-1 promoter despite the normal activation of the MAP kinases ERK-1 and ERK-2. Thus the cell lines described here provide a unique system with which to further define the pathways for Gab-1-dependent egr-1 expression.

Gab-1 also appears to be uniquely capable of enhancing IGF-1-stimulated mitogenesis. These results are supported by data that indicate that the overexpression of Gab-1 is capable of enhancing anchorage-dependent and -independent growth rates (18). In addition, Holgado-Madruga et al. (22) have shown that PC-12 cells overexpressing Gab-1 had a reduced requirement for the amount of nerve growth factor necessary to prevent apoptosis. Mutants of Gab-1 lacking PI 3-kinase binding sites exhibited increased levels of apoptosis, indicating that Gab-1-associated PI 3-kinase activity was required for the inhibition of apoptosis. In addition, the overexpression of Gab-1 in several cell systems leads to increased transforming potential indicating that Gab-1 may have a profound effect on regulation of the cell cycle. Taken together, these data indicate that Gab-1 is capable of both enhancing cell growth and promoting cell survival through the prevention of apoptosis.

The absence of alterations in MAP kinase activity in KO, KO*IRS-1, and KO+Gab-1 fibroblasts suggests that many IRS family members may not be required for the normal activation of the Ras/MAP kinase pathway. The role of Gab-1 in MAP kinase activation may differ depending on the cell type. Several groups have reported differential activation of the MAP kinases in several cell systems (18, 21, 36). For example, Holgado-Madruga et al. (18) have found that Gab-1 and SOS form separate complexes with Grb-2 indicating that Gab-1 may compete with SOS for Grb-2 binding. In this way, Gab-1 may potentially regulate the activation of the MAP kinase pathway. The absence of IGF-1-stimulated Gab-1 phosphorylation may potentially negate this effect and allow for the normal activation of the MAP kinase pathway. Alternatively, the level of overexpression of Gab-1 in our system is not sufficient to compete with SOS for Grb-2 binding, and therefore, no alterations in MAP kinase activation are observed.

In conclusion, we have demonstrated that Gab-1 is not phosphorylated by the IGF-1 receptor, but overexpression of Gab-1 in IRS-1-deficient cells can restore the IGF-1-stimulated expression of the immediate-early gene egr-1 and markedly enhances the ability of the cells to enter S-phase. Further studies are required to determine the exact mechanism by which Gab-1 participates in the regulation of these biological end points.

Acknowledgments—We thank T. L. Azar, C. Remillard, and J. Konigsberg for secretarial assistance.

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