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Expression of the art/trs Protein of HIV and Study of Its Role in Viral Envelope Synthesis

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The art/trs transactivator protein of human immunodeficiency virus (HIV) was expressed in mammalian cells as a 19-kilodalton protein that was immunoreactive with sera from HIV-infected patients. Separate plasmids encoding the art/trs protein, the tat protein, or the envelope glycoprotein gp120 were used to demonstrate that both art/trs and tat are absolutely required for the synthesis of gp120 from its cognate messenger RNA. In addition, both the tat and art/trs proteins influence the level of envelope RNA. The results suggest that art/trs and tat may be ideal targets for potential anti-HIV agents in AIDS therapy.

HUMAN IMMUNODEFICIENCY VIRUS (HIV), the cause of the acquired immune deficiency syndrome (AIDS), differs from most known retroviruses in the complexity of its genetic organization. In addition to the long terminal repeats (LTRs) and the *gag*, *pol*, and *env* genes, HIV has several genes that contribute to a system of genetic regulation far more complex than in most retroviruses. Among these are two genes, *tat* and *art/trs*, that are both required for viral replication in vitro (*1–3*). In addition, both genes code for "transactivators" that stimulate expression of other HIV genes. We will refer to the genes as *tat* and *art/trs* and the gene products as tat and art/trs. The *tat* gene product has been expressed as a 14-kD protein in both bacterial (*4, 5*) and mammalian (*6–8*) systems, but the *art/trs* gene product has not previously been identified. One important viral gene regulated by *tat* and *art/trs* is the *env* gene, which encodes the envelope glycoproteins gp120 and gp41. As a component of the viral envelope, gp120 is crucial to the interaction of the virus with its cellular receptor (*9, 10*) and may also contribute to the cytopathogenicity of HIV through its involvement in syncytium formation (*11*). It has been reported that the degree of cytopathic effect of the virus directly correlates with the amount of viral envelope protein synthesized by an infected cell (*8*). The mechanisms by which *tat* and *art/trs* regulate *env* gene expression are not clearly understood. Both tat and art/trs appear to act, at least in part, post-transcriptionally. In *tat* or *art/trs* defective proviral mutants there appears to be substantial viral RNA produced after proviral transfection, although viral protein levels are greatly reduced (*3, 8, 12*), suggesting the involvement of translational control. The *tat* gene product appears to have a bimodal function. In several systems, *tat*-mediated increase of test proteins was greater than can be accounted for by increases in the corresponding messenger RNA (mRNA). This has been interpreted to mean that translational efficiency is an important component of *tat*-mediated transactivation [(*6, 13*); for a review see (*14*)]. The relative contributions of mRNA levels versus translational efficiency in transactivation may depend on the experimental system used. For example, cell type–specific factors may play an important role in determining the final level of transactivation (*6*).

It has been difficult to assign precise roles to the tat and art/trs proteins in part because the two genes overlap (in different reading frames) with each other and with the envelope gene (see Fig. 1). Studies of *tat* or *art/trs* proviral deletion mutants have been difficult to interpret because of the possibility of more than one functional unit being altered simultaneously and because other viral genes may influence the results. Studies that examine the effects of *tat* or *art/trs* on the HIV LTR-directed synthesis of heterologous gene products may not be valid models if translational regulation that relies on mRNA structure is involved. In the study reported here, we investigated the regulation of gp120 synthesis by tat and art/trs using the simplified approach of introducing the separately cloned *env*, *tat*, and *art/trs* genes fused to the HIV LTR into mammalian cells in culture and assessing the contribution of these genes individually and in combination.

Previous studies with *art/trs* have been hampered by the inability to detect the gene product in infected cells or in cells transfected with proviruses. To achieve high levels of expression of *tat* and art/trs, we cloned the *art/trs* and *tat* coding regions derived separately from the viral complementary DNA (cDNA) clone pCV-1 (*15*) (Fig. 1B) into the plasmid pCEV, which utilizes the HIV LTR as a promoter and RNA processing signals from SV40 to express the inserted DNA as a functional mRNA (Fig. 1C). The *art/trs* construction (pART) is capable of expressing only art/trs and not tat because the ATG start codon and much of the first coding exon for *tat* have been deleted. The *tat* construction (pTAT) is capable of expressing only tat and not art/trs because it does not contain sequences following the Bam HI site in the coding region previously shown to be required for *art/trs* function (*3*). Cos-7 cells were transfected with pART or pTAT and protein extracts were made 48 hours later and analyzed by Western blot by using pooled serum samples from HIV-infected patients. Figure 2A shows that a protein of approximately 19 kD is apparent only in pART-transfected cells and not in mock transfected cells or in cells transfected with pTAT. The 19-kD protein does not react with normal human serum. We conclude that this protein is the product of the *art/trs* gene of HIV. The calculated molecular size of art/trs based on the deduced amino acid sequence is approximately 13.6 kD. The higher apparent molecular size that we observe may be due to the high proline content of the protein, which is thought to decrease the mobility of certain proteins in SDS-acrylamide gels (*4, 16*). These results show that the *art/trs* gene can be expressed at high levels as a 19-kD protein that is immunoreactive with sera from HIV seropositive patients.

Because we were unable to detect *tat* protein by Western blot, expression of *tat* was monitored by measuring its ability to transactivate the HIV LTR fused to the chloramphenicol acetyltransferase (CAT) gene. The plasmid pLTR-CAT (Fig. 1C) was cotransfected with pTAT or pART and the level of CAT activity was measured in the protein extract. Increased CAT activity over control levels is indicative of *tat* expression (*17–19*). Figure 2B shows that transactivation of the HIV LTR occurs only when pTAT is transfected, indicating that pTAT expresses the tat protein. Figure 2 also confirms that only the expected viral gene products are made from the *tat* and *art/trs* plasmids. When pART is cotransfected with

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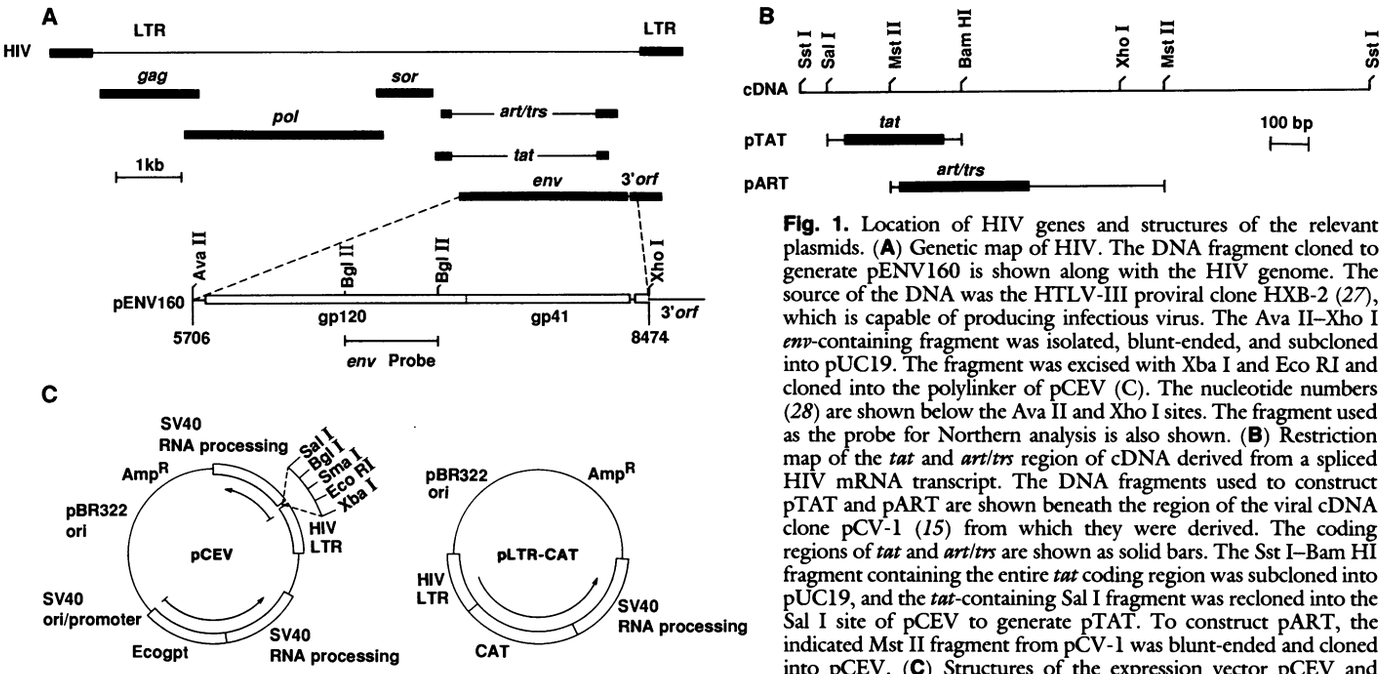

Fig. 1. Location of HIV genes and structures of the relevant plasmids. (**A**) Genetic map of HIV. The DNA fragment cloned to generate pENV160 is shown along with the HIV genome. The source of the DNA was the HTLV-III proviral clone HXB-2 (*27*), which is capable of producing infectious virus. The Ava II–Xho I *env*-containing fragment was isolated, blunt-ended, and subcloned into pUC19. The fragment was excised with Xba I and Eco RI and cloned into the polylinker of pCEV (C). The nucleotide numbers (*28*) are shown below the Ava II and Xho I sites. The fragment used as the probe for Northern analysis is also shown. (**B**) Restriction map of the *tat* and *art/trs* region of cDNA derived from a spliced HIV mRNA transcript. The DNA fragments used to construct pTAT and pART are shown beneath the region of the viral cDNA clone pCV-1 (*15*) from which they were derived. The coding regions of *tat* and *art/trs* are shown as solid bars. The Sst I–Bam HI fragment containing the entire *tat* coding region was subcloned into pUC19, and the *tat*-containing Sal I fragment was recloned into the Sal I site of pCEV to generate pTAT. To construct pART, the indicated Mst II fragment from pCV-1 was blunt-ended and cloned into pCEV. (**C**) Structures of the expression vector pCEV and pLTR-CAT. To construct pCEV, an expression cassette including the HIV LTR from HXB-2 through nucleotide +80, a synthetic polylinker containing the indicated restriction enzyme sites, and the SV40 RNA processing signals derived from pSV2neo (*29*) was cloned into the Eco RI site of pSV2gpt (*30*). pLTR-CAT was constructed by replacing the SV40 promoter/enhancer region of pSV2-CAT (*31*) with the HIV LTR through nucleotide +258. The transcriptional units of both plasmids are indicated by arrows.

pLTR-CAT there is no transactivation, indicating that no detectable tat is present (Fig. 2B). Similarly, transfection of cos cells with pTAT does not produce the 19-kD immunoreactive art/trs protein (Fig. 2A).

To examine the effects of tat and art/trs on gp120 synthesis, we developed a cotransfection protocol using various combinations of *env*, *tat*, and *art/trs* plasmids. pENV160 (Fig. 1A), which contains the entire coding region of the envelope gene fused to the HIV LTR, was used to express gp120. The gp160 precursor protein and the gp41 transmembrane protein are also encoded by pENV160; our methods of gp120 analysis, however, do not reproducibly detect these proteins. Furthermore, this plasmid does not contain the first coding exons of *tat* and *art/trs*, and is therefore incapable of expressing these proteins. Cells were harvested 48 hours after transfection and analyzed by Western blot for gp120 and art/trs production with pooled sera from HIV-infected patients. Each transfection also included pLTR-CAT to enable measurement of tat by transactivation of the HIV LTR.

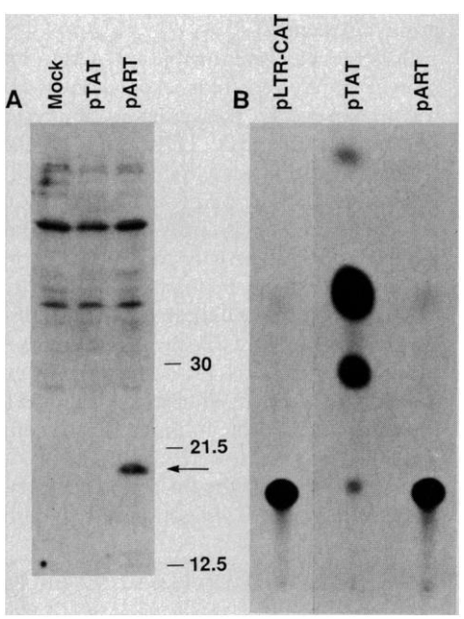

Fig. 2. Expression of the *art/trs* and *tat* gene products. Cos-7 cells were plated at a density of 2×10^6 cells per plate and transfected 24 hours later by the calcium phosphate coprecipitation method (*32*). (**A**) Western blot of total cell proteins obtained 48 hours after mock transfection (no DNA), transfection with 10 μg of pTAT, or with 10 μg of pART. The pTAT and pART transfections also included 5 μg of pLTR-CAT to allow measurement of tat activity. Proteins were separated on a 15% SDS-polyacrylamide gel, transferred to nitrocellulose, and allowed to react with a pool of sera from HIV-infected patients. An ^{125}I-labeled goat antiserum to human second antibody was then used to detect immunoreactive proteins (*33*). The positions of molecular weight markers are shown (size in kilodaltons), and the arrow indicates the position of the 19-kD protein present only in the pART-transfected cells. (**B**) CAT assays (*31*) performed on extracts of transfected cos-7 cells. The pLTR-CAT lane represents transfection with 5 μg of pLTR-CAT and 10 μg of pUC19 DNA to equalize the total amount of transfected DNA. The pTAT and pART lanes, from the same experiment as in (A), represent transfections with 5 μg of pLTR-CAT and 10 μg of either pTAT or pART.

Figure 3A shows that when pENV160 was transfected into cos-7 cells, no gp120 was detected. When pENV160 was cotransfected with pTAT, gp120 was still not seen, even though functional tat protein was present (Fig. 3B), indicating that tat alone is incapable of transactivating gp120 synthesis. When pART was cotransfected with pENV160, art/trs protein was expressed but again no gp120 was detected (Fig. 3A). In contrast, when pENV160 was cotransfected with both pTAT and pART, there was a strong band corresponding to gp120. Note that the amount of art/trs is increased in the presence of tat, presumably because of tat-mediated transactivation of the LTR of pART. To confirm the identity of the gp120 band and to detect possible low levels of gp120, we incubated an identical Western blot with a murine monoclonal antibody that reacts with purified gp120 (*20*). This result (Fig. 3C) confirms the pattern of gp120 expression observed in Fig. 3A. In addition, a long exposure of the same blot (lower portion of Fig. 3C) reveals no gp120 in the absence of art/trs or tat, allowing us to conclude that both tat and art/trs must be present for gp120 synthesis to occur when the HIV LTR is used as the promoter. The increase in gp120 is not simply due to increased transactivation of the LTR with art/trs present because the transactivation levels measured by CAT assay are no higher

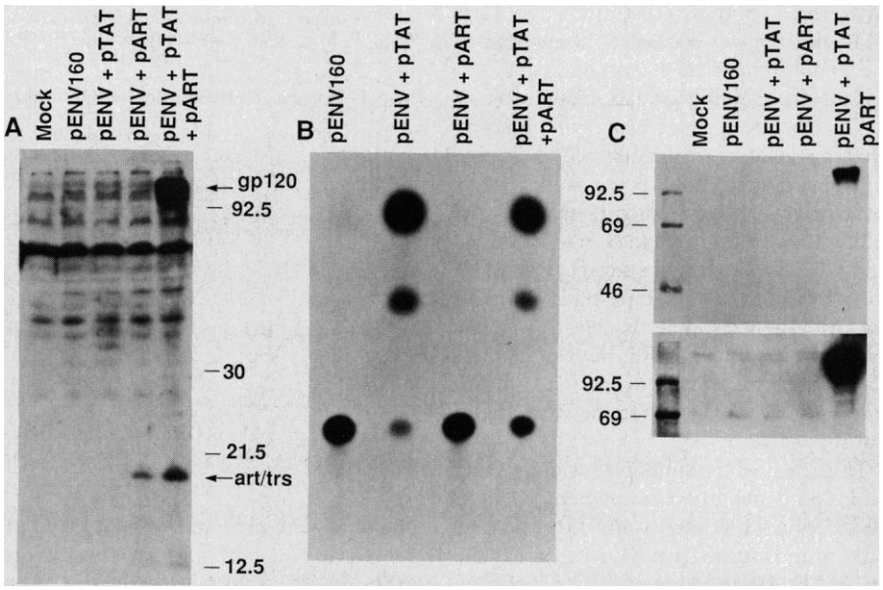

Fig. 3. gp120 synthesis requires the *art/trs* and *tat* gene products. Cos-7 cells were transfected as described in Fig. 2 with 10 μg of each of the indicated plasmids. Five micrograms of pLTR-CAT was included in each transfection, and the total amount of DNA was held constant by the addition of pUC19 DNA. The extracts were analyzed 48 hours after transfection for gp120, art/trs, and tat activity. (**A**) Western blot of total cell proteins separated on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and reacted with a pool of HIV-infected patient sera. An ^{125}I-labeled goat antiserum to human second antibody was used to detect immunoreactive proteins. The positions of molecular weight markers are shown (size in kilodaltons), and the positions of the art/trs and gp120 proteins are marked with arrows. (**B**) CAT assays (*31*) performed on the same extracts as in (A). (**C**) An identical blot to (A) but reacted with a monoclonal antibody that reacts with purified gp120 (*20*). A second ^{125}I-labeled goat antiserum to mouse immunoglobulin was used to visualize gp120. The upper panel represents an 8-hour exposure and the lower panel, a 2-week exposure of the same blot. The arrow indicates the position of gp120. ^{14}C-labeled molecular weight markers are indicated. Only relevant portions of the blot are shown.

with pTAT plus pART than with pTAT alone (Fig. 3B). Because other genes, both viral (for example, *art/trs*) and heterologous [CAT and interleukin-2; see (*13, 17–19*)], can be expressed from the HIV LTR without the presence of art/trs or tat, this result implies the existence of a specific intrinsic block of gp120 synthesis.

To investigate the levels of gene expression at which tat and art/trs transactivate gp120 synthesis, we performed a Northern blot analysis using total cellular RNA derived from cells transfected as above and hybridized the blot to a DNA probe to detect envelope sequences. The probe was a ^{32}P-labeled internal Bgl II fragment of *env* (Fig. 1A) which does not contain any sequences in common with *tat* or *art/trs*. Figure 4 shows that *env* RNA is detectable in every case where pENV160 was transfected. The major band is approximately the expected size (3.5 kb) for the *env* mRNA transcribed from pENV160; some minor bands of unknown origin are also present. In the presence of tat, the steady-state level of *env* RNA is increased 15- to 20-fold. This is consistent with studies of fusions between the HIV LTR and heterologous genes in which tat increases RNA levels to a similar extent (*6, 7, 13, 21*). The art/trs protein alone, however, has essentially no effect on the level of *env* RNA. When both tat and art/trs are present, there is a further increase (approximately threefold) in *env* RNA content over that observed with tat alone, indicating that art/trs can influence RNA levels in the presence of tat. From these experiments it is not clear whether the increases in *env* RNA are the result of a higher transcription rate, increased stability of the mRNA, or both. Irrespective of the mechanism that generates higher steady-state RNA levels, this increase in RNA clearly cannot account for the dramatic increase in gp120 protein shown in Fig. 3. Surprisingly, even though there is *env* RNA present in all cases (a high level in the presence of tat), only when both tat and art/trs are present can the RNA give rise to gp120 (Fig. 3, A and C). We have thus observed two effects of tat and art/trs on the expression of the *env* gene. The combination of tat and art/trs appears to be necessary for translation of *env* RNA and each increases the steady-state level of *env* RNA, although art/trs requires the presence of tat for its effect.

The data presented here suggest that there is an intrinsic translational block in gp120 synthesis and that tat and art/trs circumvent this block and allow *env* RNA to be translated. The effect that we observe of art/trs on gp120 synthesis is unlikely to be due to alterations in *env* mRNA splicing patterns caused by art/trs as has been suggested by studies with proviral deletion mutants (*8*). In our pENV160 construction, the viral splice donor site at nucleotide 289, common to all known HIV mRNAs, is absent; the possible *env* splice acceptor sites at nucleotides 5359 or 5558 (*22*) are both absent as well. In addition, we see no difference in the size of *env* RNA in the presence or absence of art/trs (Fig. 4).

What is the nature of the translational block in gp120 synthesis? At least one element must be provided by the envelope sequences themselves because heterologous genes can be expressed from the HIV LTR without the presence of tat or art/trs, indicating that there is no comparable inhibition in these cases. However, it is unlikely to be only envelope sequences that cause the translational inhibition because gp120 has been expressed with the use of heterologous promoters in several systems (*23–26*), although in one case a fusion protein was expressed which lacked a small portion of the natural envelope sequences. In experiments with the SV40 early promotor fused to the HIV envelope gene we found that gp120 can be expressed from a heterologous promoter; in this case, its synthesis was not regulated by tat or art/trs.

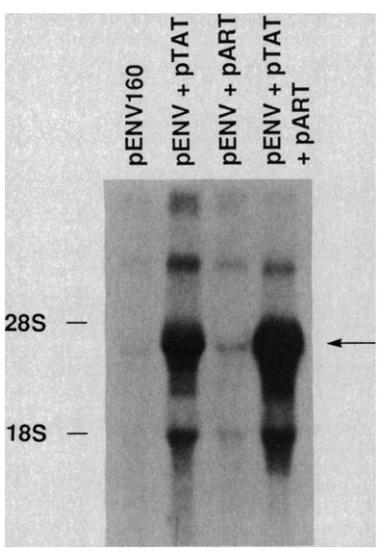

Fig. 4. Levels of *env* RNA in cells transfected with pENV160, pART, and pTAT plasmids. Transfections with the indicated plasmids were performed as in Fig. 2 and total RNA was prepared by the hot phenol method as described (*34*). Twenty micrograms of each RNA sample were subjected to electrophoresis on a formaldehyde-agarose gel, transferred to nitrocellulose, and hybridized with a nick-translated ^{32}P-labeled DNA fragment (Fig. 1A) to detect *env* sequences. The arrow indicates the position of the 3.5-kb *env* RNA. The positions of the 28*S* and 18*S* ribosomal RNAs are shown.

Although other scenarios are possible, we favor the hypothesis that both the HIV LTR and the *env* sequences contribute to the establishment of a translational block, possibly through the formation of secondary RNA structure or the creation of a binding site for a cellular translational repressor protein. The tat and art/trs proteins may interact with the RNA, relieving the translational inhibition and, in the process, stabilizing the *env* RNA so that higher steady-state levels are achieved when these proteins are present. Although tat alone is able to increase *env* RNA levels either by increasing transcription or stability of the RNA, both tat and art/trs are required for the synthesis of gp120.

Thus, using a simplified system that avoids potential problems inherent in proviral deletion experiments and heterologous gene fusion studies, we have expressed the tat and art/trs proteins and have obtained clear evidence that both are necessary for gp120 synthesis. The complete lack of gp120 synthesis in the absence of tat and art/trs observed here suggests that these proteins would be attractive targets for therapeutic agents for AIDS. The system described here may provide a simple method for testing such agents.

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Ras p21 as a Potential Mediator of Insulin Action in *Xenopus* Oocytes

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The oncogene protein product (p21) of the *ras* gene has been implicated in mediating the effects of a variety of growth factors and hormones. Microinjection of monoclonal antibody 6B7, which is directed against a synthetic peptide corresponding to a highly conserved region of p21 (amino acids 29 to 44) required for p21 function, specifically inhibited *Xenopus* oocyte maturation induced by incubation with insulin. The inhibition was dose-dependent and specific since (i) the same antibody had no effect on progesterone-induced maturation, (ii) immunoprecipitation and Western blotting indicated that the antibody recognized a single protein of molecular weight 21,000 in oocyte extracts, and (iii) inhibition was not observed with identical concentrations of normal immunoglobulin. Thus, p21 appears to be involved in mediating insulin-induced maturation of *Xenopus* oocytes. Furthermore, the mechanism may involve phosphorylation of p21, as p21 was found to be a substrate of the insulin receptor kinase.

THE PROTEIN PRODUCTS OF THE *ras* cellular proto-oncogenes are a family with a molecular weight of 21,000; they are membrane-bound, guanine nucleotide–binding proteins that are thought to function in growth control of cells (*1–10*). In particular, several studies have suggested indirectly that a member of the p21 family mediates the ability of cells to respond to insulin. First, insulin as well as epidermal growth factor stimulate the phosphorylation and guanine nucleotide–binding activity of H-*ras* p21 in cells transformed with Harvey murine sarcoma virus (*11*). Second, some of the effects of insulin on adipose cells are dependent on the functioning of a guanosine 5′-triphosphate (GTP)–binding protein (*12, 13*). Third, p21 has been shown to regulate membrane ruffling and possibly phospholipase C (*14*) or A_2 (*15*) activity. Similarly, insulin has been shown to regulate membrane ruffling (*16*) and to activate a specific phospholipase C (*17*).

To test directly whether p21 is a mediator of insulin action, we used the *Xenopus* oocyte system. *Xenopus* oocytes can be induced to mature in vitro by incubation with insulin (*18*) or progesterone (*19*). These two hormones exert their effects directly by binding to distinct receptors and triggering different signaling mechanisms (*18, 20, 21*). The activation of an intrinsic tyrosine kinase activity of the β subunit of the insulin receptor is important in mediating insulin's effects on *Xenopus* oocytes (*22*) and mammalian cells (*23, 24*). In contrast, progesterone induces oocyte maturation by inhibiting adenylate cyclase and thus lowering adenosine 3′,5′-monophosphate (cAMP) levels (*25–29*). Recently, microinjection of p21 into *Xenopus* oocytes was also shown to induce oocyte maturation (*4*). Unlike progesterone, the effect of p21 was not accompanied by any change in the intracellular levels of cAMP (*4*). These results therefore suggested that p21 might mediate the effects of insulin.

To further study the role of p21 in mediating insulin effects, we have made use of monoclonal antibody 6B7 (anti-p21), which is directed against a synthetic peptide corresponding to residues 29 to 44 of p21 (*30*). This region is highly conserved among *ras* proteins but is not found in any other characterized GTP-binding proteins (*31,*

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