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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32). By site-directed mutagenesis, this region has also been shown to be important for p21 function (31). This antibody is highly specific for p21, as it has been demonstrated to react with the protein products of H-, N-, and K-*ras* but not with two other GTP-binding proteins (elongation factor EF-Tu and transducin) (33, 34).

To verify the specificity of the anti-p21 in the *Xenopus* system, extracts of oocytes were prepared, immunoprecipitated with the antibody, and analyzed on Western blots as described in Fig. 1. This combined procedure of immunoprecipitation followed by Western blotting is more sensitive than either procedure performed separately (35). This analysis revealed a single specific band of $M_r \approx 21{,}000$, corresponding to the known size of mammalian *ras* protein (Fig. 1). The band of M_r = 30,000 present in both the control lane and the lane treated with anti-p21 represents immunoglobulin light chain, which is released during the procedure (see legend to Fig. 1). In summary, these results demonstrate that in oocyte extracts, antibody 6B7 specifically recognizes a single protein with the expected molecular weight of *Xenopus ras* p21.

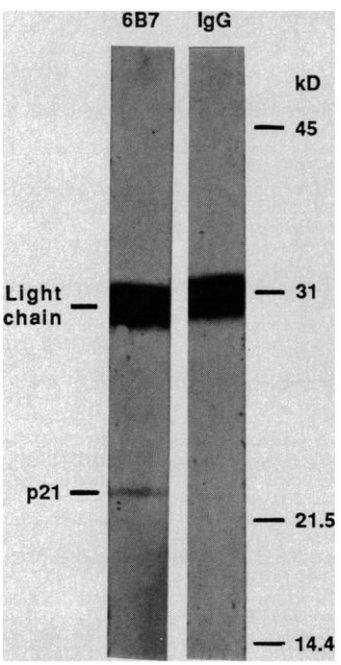

Fig. 1. Immunoprecipitation and Western blotting of oocyte extracts. For the Western analysis, 20 oocytes per antibody sample (10 μg of antibody) were lysed in 0.5% NP40 and 0.5% deoxycholate tris-saline and centrifuged. The supernatants were reacted with anti-p21 (6B7) coupled to Sepharose beads. Protein was eluted with 2.0% SDS and then reduced with 0.1*M* dithiothreitol after centrifuging out the beads. Some immunoglobulin light chain (M_r = 30,000) is released during this procedure. Samples were separated by electrophoresis through an SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with either control mouse IgG (IgG) or antibody 6B7 (46). The blots were then developed with peroxidase-coupled antibody to mouse IgG (30).

Injection of human *ras* protein into *Xenopus* oocytes induces maturation, as assayed by the appearance of a white spot in the pigmented half of the oocyte, by the breakdown of the germinal vesicle, and by the appearance of a meiotic spindle (4). To determine whether antibody 6B7 could block oocyte maturation induced by p21 itself, we incubated v-H-*ras* Val^{12} protein and antibody for 1 hour and then injected the mixture into the cytoplasm of *Xenopus* oocytes. When p21 (at an intracellular concentration of 2.2 μ*M*) was injected alone or with normal mouse immunoglobulin G (IgG; 2.6 μ*M*) into oocytes, 96 and 86%, respectively, of the oocytes matured. In contrast, when p21 (2.2 μ*M*) was mixed with anti-p21 (2.7 μ*M*) and then injected into oocytes, only 19% of the oocytes matured. Thus, 6B7 inhibited p21-induced maturation by 80%.

The effect of anti-p21 on the ability of insulin and progesterone to induce maturation was then examined. Uninjected oocytes and oocytes injected with control IgG (13 μ*M*) responded similarly in terms of germinal vesicle breakdown (GVBD) to 1.0 μ*M* progesterone and 0.5 μ*M* insulin (Table 1), indicating that the injection of antibody itself does not affect oocyte maturation. Oocytes that were injected with the anti-p21 (6B7) showed a 95% inhibition in their response to insulin, but there was no inhibition of the progesterone effect. These results were confirmed at insulin and progesterone concentrations ranging between 0.04 and 1.0 μ*M* in four separate experiments, two of which are summarized in Table 1. In summary, anti-p21 specifically inhibited the insulin response and not the progesterone response of *Xenopus* oocytes. These results demonstrate that the antibody did not inhibit maturation per se and suggest that p21 is part of the normal cascade of reactions of insulin-mediated, but not progesterone-mediated, oocyte maturation.

Since the concentrations of insulin required to elicit oocyte maturation are relatively high compared to that in other tissues, it is possible that the effects of insulin are mediated through a related receptor, the receptor for insulin-like growth factor I (18). This receptor has a tyrosine-specific kinase activity that is immunologically indistinguishable from that of the insulin receptor (36). The two receptors also share 84% sequence homology in the kinase domain (37). In either case, a highly related tyrosine kinase activity is involved in mediating the insulin-induced maturation of oocytes.

Inhibition of the insulin (0.8 μ*M*) response was dependent on antibody concentration (Fig. 2) and was nearly complete at intracellular anti-p21 concentrations of 13 and 5 μ*M*. At these concentrations only 6 and 18% (respectively) of the injected oocytes, compared to 73% of the uninjected oocytes, matured. There was no inhibition of maturation at 0.3 μ*M* anti-p21, as 70% of the oocytes matured. In contrast, injected control IgG did not significantly inhibit the oocyte response at any of the concentrations tested.

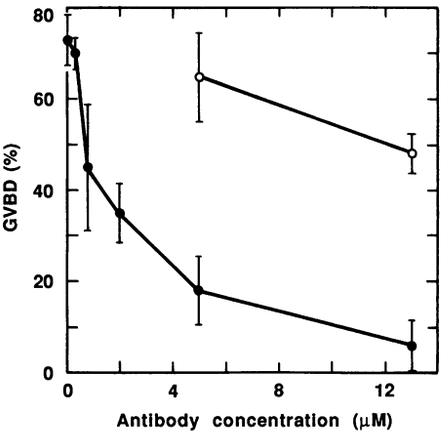

Fig. 2. Dependence of inhibition of the insulin response on antibody concentration. Oocytes were obtained and treated, and antibody concentrations were calculated, as described in the legend to Table 1. Injected oocytes (about 30 at each concentration) were divided into three equivalent groups, and each group was incubated separately in 1 ml of Barth's medium containing 0.1% bovine serum albumin and 0.8 μ*M* insulin. The graphed results represent the mean GVBD ± standard error for the triplicate groups. Note that in contrast to antibody 6B7, IgG did not inhibit the insulin response. The slight decrease in the insulin response observed for oocytes injected with IgG at the highest concentration (13 μ*M*) is probably not statistically significant; indeed, in other experiments (see Table 1), a proportion of uninjected oocytes, similar to that of oocytes injected with IgG, mature in response to both hormones. ●, Antibody to p21; ○, mouse IgG.

Progesterone induces oocyte maturation by causing a decrease in cAMP levels via inhibition of the oocyte adenylate cyclase (25–29). Consistent with these findings, cholera toxin, which causes an increase in cAMP levels, inhibits progesterone-induced maturation (38, 39). In contrast, the effects of insulin, such as changes in intracellular *p*H and phosphorylation of the ribosomal protein *S6*, persist even in the presence of cholera toxin (40). These results suggest that insulin and progesterone exert their effects on oocytes through separate biochemical pathways. Like insulin, *Xenopus* p21 appears to function as part of a biochemical pathway that does not involve fluctuations in cAMP levels. Birchmeier *et al.* (4) have reported that the effects of p21 on *Xenopus* oocytes do not involve significant alterations of cAMP

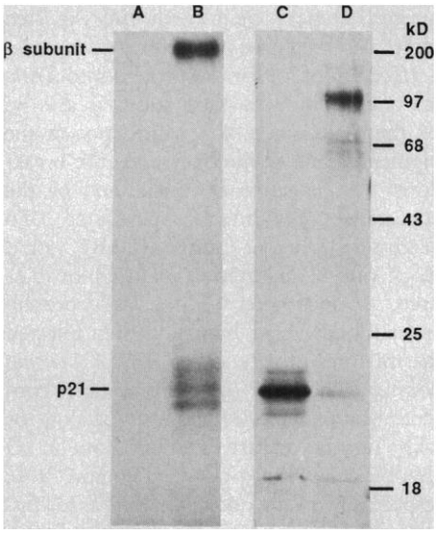

Fig. 3. Phosphorylation of p21 by purified insulin receptor. Wild-type human N-*ras* p21 (5 μg, produced in *Escherichia coli*) was incubated with either buffer (lane A) or 50 ng of purified human placental insulin receptor (lane B) in the presence of 0.1% Triton X-100, 1 μ*M* insulin, 5 m*M* $MgCl_2$, 2 m*M* $MnCl_2$, 10 μ*M* [γ-^{32}P]ATP (10 Ci/mmol). After 1 hour at 24°C the reaction mixture was separated by electrophoresis through a 12.5% polyacrylamide–SDS gel. The autoradiogram of the gel is shown. For the experiment shown in lanes C and D, p21 was phosphorylated by the insulin receptor kinase as described above, separated by electrophoresis through a 10% polyacrylamide–SDS gel, transferred to a nitrocellulose filter, and probed with either monoclonal antibody 6B7 (lane C) or polyclonal rabbit antibody to phosphotyrosine (lane D) (*47*). The Western blots were developed with the appropriate antibody to IgG coupled with alkaline phosphatase (Promega). Molecular sizes were determined by the inclusion of prestained molecular weight marker proteins (BRL). These markers correspond to lanes C and D only. The tyrosine phosphorylated band at 97 kD in lane D represents the autophosphorylated β subunit of the receptor.

levels and are not all inhibited by cholera toxin or phosphodiesterase inhibitor. In addition, p21 and progesterone were found to act synergistically in stimulating maturation (*4*), thus further indicating that p21 and progesterone act through separate pathways. Other laboratories have also found that p21 function in mammalian cells does not involve cAMP fluctuations (*3, 41*). In yeast, the one eukaryotic system where *ras* proteins have been shown to activate adenylate cyclase (*42, 43*), it appears that p21 probably exerts its effects indirectly through as yet unidentified proteins (*4*). Our finding that a monoclonal antibody to p21 specifically inhibits insulin- but not progesterone-induced maturation further supports the hypothesis that insulin and progesterone induce oocyte maturation by acting through separate pathways and indicates that *ras* p21 is part of the pathway affected by insulin.

Recent studies by Sadler *et al.* (*44*) have indicated that microinjection of oocytes with another monoclonal antibody (238) directed against the v-H-*ras* protein accelerated progesterone-induced maturation. However, this antibody was reported to have no effect on p21-induced responses in mammalian cells (*7, 8*).

It is possible that *ras* p21 indirectly affects enzymes involved in pathways interconnected with the insulin pathway; however, in the simplest model consistent with our results, p21 is a part of the cascade of reactions mediating insulin-induced oocyte maturation. Presumably the insulin receptor's tyrosine kinase, stimulated by the binding of insulin, interacts directly or through as yet unidentified proteins with p21. In vitro studies indicate that purified insulin receptor can phosphorylate p21 (Fig. 3, lanes A and B). This phosphorylation occurs on a tyrosine residue since antibodies specific to phosphotyrosine react on a Western blot with phosphorylated p21 (Fig. 3, lanes C and D). To determine the significance of these in vitro phosphorylations, additional studies will be required to determine whether a similar phosphorylation occurs in vivo. A phosphorylation of p21 on a tyrosine residue could then regulate its GTPase activity, thereby affecting its interaction with a phospholipase activity (*9, 14–17*), and/or its ability to elicit known responses to insulin. With the system described here, it should be possible to further explore the role of p21 in mediating the various biological effects of insulin.

Table 1. Monoclonal antibody 6B7 inhibits insulin-induced, but not progesterone-induced, maturation of *Xenopus* oocytes. Stage VI oocytes from female *Xenopus laevis* (Nasco) primed with gonadotropin from pregnant mares' serum (Sigma) were manually dissected from their ovarian follicle, placed in Barth's medium (10 m*M* tris-HCl, 8.8 m*M* NaCl, 2.4 m*M* $NaHCO_3$, 0.8 m*M* $MgSO_4$, 1 m*M* $CaCl_2$, 10 m*M* Hepes, *p*H 7.6), and microinjected into the cytoplasm with the indicated antibody (50 nl per oocyte). Injected oocytes were incubated for 5 hours at 19°C, and healthy oocytes (about 20) then were transferred to Barth's medium (10 oocytes per milliliter) containing 0.1% bovine serum albumin and the indicated hormone. After an overnight incubation (16 hours) at 19°C, oocytes were analyzed for maturation by the appearance of a white spot in the pigmented animal pole. This white spot has been shown to correlate with GVBD. Without hormone, there were no mature oocytes. Antibody concentrations are the final concentrations in the oocyte. Protein concentrations were calculated by assuming a size of 150 kD for the antibodies and an intracellular volume of 1 μl per oocyte, half of which was assumed to be inaccessible to microinjected protein because of high yolk content (*45*). For a total protein content of 280 μg per oocyte (*45*), the injected antibodies in experiments 1 and 2 represent less than 0.4 and 0.2%, respectively, of the total cellular protein.

Microinjected antibody	Progesterone (μ*M*)				Insulin (μ*M*)					
	1	0.2	0.1	0.04	1	0.5	0.2	0.1	0.05	0.04
	Experiment 1 (% positive for maturation)									
None	86		87			74		59	16	
Mouse IgG (13 μ*M*)	94		80			77		27	23	
Anti-p21 (6B7) (13 μ*M*)	94		100			4		4	0	
	Experiment 2 (% positive for maturation)									
Mouse IgG (6.5 μ*M*)	100	73		9	73		43			44
Anti-p21 (6B7) (5.6 μ*M*)	89	71		0	15		27			0

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Chemical Identification of a Tumor-Derived Angiogenic Factor

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Neoplasms produce substances that induce blood vessel formation (angiogenesis). Fractions from ethanol extracts of the Walker 256 carcinoma were isolated by silica column chromatography and C_{18} reversed-phase high-performance liquid chromatography. Two of the isolated fractions induced neovascularization when tested in the rabbit corneal micropocket assay. One of the fractions was identified as nicotinamide by desorption–electron impact mass spectrometry, nuclear magnetic resonance spectroscopy, and gas chromatography–mass spectrometry. The second active fraction contained nicotinamide as part of a more complex, as yet unidentified, molecular arrangement. Microgram quantities of commercial nicotinamide induced neovascularization in the corneal micropocket assay and in the chick chorioallantoic membrane assay.

ANGIOGENESIS IS THE PROCESS whereby blood vessels proliferate. The process is a normal part of embryonic development, wound healing, and limb and organ regeneration. It plays a facilitative role in disease states such as cancer (*1*) and numerous retinopathies (*2*). Some neoplasms release factors that induce angiogenesis in experimental models (*1*). Even normal tissue at risk for malignancy (preneoplastic) bears an increased capacity to induce angiogenesis (*3*). Well-characterized proteinaceous substances that are angiogenic include basic fibroblast growth factor (FGF) (*4*), α-transforming growth factor (*5*), and angiogenin (*6*). No low molecular weight, organic, tumor-derived angiogenic substances have yet been identified.

The Walker 256 carcinoma has served as a classic source of angiogenic factors (*1, 7*). Low molecular weight extracts with light absorbance maxima at 260 nm were observed by Vallee *et al.* (*7*) and by Fenselau *et al.* (*8*). The extract described by Fenselau *et al.* (*8*) was partially purified by silica gel chromatography. It stimulated blood vessel proliferation in two models of angiogenesis in vivo, the rabbit corneal micropocket assay and the chick chorioallantoic membrane. It also stimulated endothelial cell growth in culture.

We have isolated two active moieties from similar extracts. One was identified as nicotinamide, a vitamin and a component of the ubiquitous cofactors nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP). The other active component was a more complex molecular arrangement that contained nicotinamide (or derivatives). Walker 256 carcinoma cell homogenates were extracted, and the extracts were run on sequential silica gel columns (*8, 9*). The capacity of fractions to stimulate endothelial cell proliferation was determined as detailed elsewhere (*10*). The active eluate from the silica column was further fractionated by reversed-phase high-performance liquid chromatography (HPLC). Three major and numerous minor peaks were obtained (*11*). The major peaks were examined for angiogenic activity in the rabbit corneal micropocket assay (*12*). This assay involves surgically implanting test material contained in a vinyl polymer pellet.

Figure 1 compares the rates of vessel growth. Material from peak 3 (P3) showed strong activity in the assay. Evidence for the structure of P3 was first derived from a desorption–electron impact mass spectrum (*13*). The spectrum was identical to that of commercial nicotinamide (Fig. 2). Comparison of HPLC retention times in two systems (*11*), ultraviolet (UV) absorbance spectra (*11*), nuclear magnetic resonance (NMR) spectrometry (*14*), and gas chromatography–mass spectrometry (*15*) provided additional agreement. All data were identical. No other organic constituents were apparent in the tumor-derived P3 material. An equivalent amount of commercial nicotinamide (40 μg per pellet) was also active in the micropocket assay, but vessel outgrowth commenced later (Fig. 1). A separate trial of nicotinamide showed activity at 20 μg per pellet. Microgram quantities of

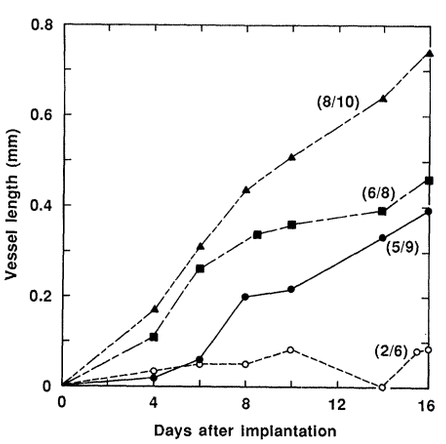

Fig. 1. Rate of vessel growth. Angiogenesis was assessed by the corneal micropocket assay in rabbits as described (*12*). Eight A_{260} units of test material were incorporated into ten 1.5-mm^3 Elvax (a vinyl polymer) pellets. Each pellet was implanted in a cornea, 1 mm from the limbus. The number and length of new vessels growing from the limbus to the pellet were determined three times weekly for 2 weeks. Points show the average vessel length. The standard error of the mean for each point was approximately 30% of its value. Parenthetical numbers indicate the number of implants eliciting any angiogenic response relative to the number evaluated. Inflamed corneas were not included in the tabulation. Nicotinamide-induced angiogenesis was noted for its lack of inflammation as assessed by stereomicroscopy. ▲, P3; ■, P2; ●, commercial nicotinamide; ○, P1.

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