termine if the structures we observed in nuclei of CIN-612 cells were indeed virions. Dot blot hybridization was performed on fractions from an isopycnic gradient purification (*17*) of HPV 31b virions produced in raft culture, and the presence of viral DNA was confirmed by Southern (DNA) blot hybridization (Fig. 4A). Lanes 6, 7, and 8 of Fig. 4A demonstrate the presence of HPV 31b DNA at low levels. From the copy number standards, we estimate the yield of viral particles to be at least 40 million per milliliter in lanes 7 and 8 (Fig. 4A). Fractions positive for HPV 31b DNA contained viral particles (Fig. 4B). The density gradient in fractions where virions were found was between 1.3 and 1.4 g/cm^3 as determined by weight. The presence of both HPV DNA and viral particles within the same fractions suggests that these are complete HPV virions, not empty capsids.

The ability to propagate papillomavirus in vitro is valuable not only for the understanding of the virus, but also for the eventual development of anti-viral treatments and the prevention of papillomavirus-induced lesions. These studies also establish the tight link between epithelial differentiation and HPV virion production. The ability of TPA to induce an increased expression of differentiation-specific markers suggests that a signal transduction pathway of epithelial differentiation has been identified. Virion production induced by phorbol esters has been described in other systems such as Epstein-Barr virus (*18*), Pichinde virus (*19*), Rift Valley fever virus (*20*), cytomegalovirus (*21*), and human immunodeficiency virus (*22*). The induction of the complete vegetative life cycle of HPVs in vitro requires both stratification at an air-liquid interface and protein kinase C (PKC) activation. In support of this hypothesis, the induction of viral particle biosynthesis by the addition of the synthetic diacylglycerol 1,2-dioctanoyl-*sn*-glycerol was observed (*23*). We believe that PKC activation of papillomavirus production is dependent on the induction of a more complex keratinocyte differentiation program and is not just a direct effect on capsid synthesis.

In this report, we described a system whereby latent infection of keratinocytes is converted into a productive infection leading to the formation of papillomavirus virions in culture. This tissue culture system will be useful in studies of the mechanisms whereby latency is maintained and terminated, and in the synthesis and assembly of papillomavirus virions.

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Rapamycin-Induced Inhibition of the 70-Kilodalton S6 Protein Kinase

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The immunosuppressant rapamycin inhibited proliferation of the H4IIEC hepatoma cell line. Rapamycin, but not its structural analog FK506, also inhibited the basal and insulin-stimulated activity of the p70 ribosomal protein S6 kinase. By contrast, insulin stimulation of the p85 Rsk S6 kinase and mitogen-activated protein (MAP) kinase activity were unaffected by drug. Rapamycin treatment of COS cells transfected with recombinant p70 S6 kinase completely inhibited the appearance of the hyperphosphorylated form of p70 S6 kinase concomitant with the inhibition of enzyme activity toward 40*S* subunits. Thus, rapamycin inhibits a signal transduction element that is necessary for the activation of p70 S6 kinase and mitogenesis but unnecessary for activation of p85 Rsk S6 kinase or MAP kinase.

Increased phosphorylation of multiple serine residues on the 40S ribosomal protein S6 numbers among the most rapid biochemical responses exhibited by cells stimulated with insulin or mitogens in vitro (*1*). Insulin or mitogen-stimulated S6 phosphorylation is catalyzed by one or both of two families of insulin or mitogen-activated S6 Ser-Thr protein kinases—the Rsk or p85 Rsk S6 kinases (*2*), and the p70 S6 kinases (*3*, *4*). Both families of S6 kinases are themselves regulated by Ser-Thr phosphorylation, although the immediate upstream regulators of the two S6 kinase families differ, at least in part. The *Xenopus* S6 kinase II, a p85 Rsk enzyme, is activated in vitro by phosphorylation with p42 MAP kinase (*5*). The MAP kinases are the dominant (perhaps only) immediate upstream activator of the p85 Rsk enzyme in situ in response to insulin or mitogens (*6*). Although the MAP kinases and cdc2 phosphorylate recombinant p70 S6 kinase in vitro in a putative regulatory

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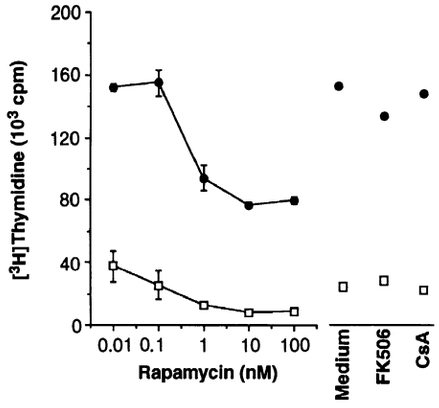

Fig. 1. Rapamycin-mediated inhibition of basal (□) and insulin-stimulated (●) proliferation of H4 rat hepatoma cells. Cells were grown to near-confluence in Swims S77 medium (Sigma) with fetal calf serum (5%) and horse serum (15%) at 37°C, 5% CO_2. Cells were cultured in serum-free medium 18 to 24 hours before assay. Serum-starved H4 cells were cultured in 96-well, flat-bottom plates (4 × 10^4 cells per well) in the absence (□) or presence (●) of 10^{-6} M insulin. CsA (100 nM), FK506 (100 nM), and rapamycin (as indicated) were added at the start of the assay. Proliferation was assessed by the incorporation of [^{3}H]thymidine during 16 hours after a 32-hour incubation. Mean ± SEM of triplicate determinations is shown, and this experiment is representative of four experiments.

domain (*7*), this phosphorylation is not sufficient to activate the p70 S6 kinase, indicating the existence of other, as yet unidentified, insulin-mitogen–activated p70 kinase–kinases (*7*).

The macrolide immunosuppressant rapamycin and its structural analog FK506 bind to the same family of intracellular receptors, termed FK506 binding proteins (FKBPs) (*8*). The complex of FK506 with FKBP binds to and inhibits the activity of calcineurin, a calcium-calmodulin–dependent Ser-Thr phosphatase (*9*). FK506, like the undecapeptide immunosuppressive agent cyclosporin A (CsA), inhibits T cell receptor–mediated events leading to lymphokine gene transcription (*10*). Rapamycin, but not FK506, inhibits lymphokine-dependent proliferation of cells at the G1 to S phase of the cell cycle (*10, 11*). The molecular target of rapamycin action has not been defined. We now demonstrate that the signal transduction pathway leading to the activation of p70 S6 kinase is selectively inhibited by rapamycin.

Rapamycin selectively inhibited the incorporation of [^{3}H]-labeled thymidine into serum-starved H4 hepatoma cells in a concentration-dependent fashion both in the presence of IC_{50}, half-maximal inhibitory concentration, of ~0.1 nM and absence of ~0.5 nM of the mitogen insulin (Fig. 1). Neither FK506 nor CsA, at 100-fold greater concentrations than those effective for rapamycin, inhibited basal or insulin-stimulated H4 proliferation (Fig. 1).

To analyze the rapamycin-mediated inhibition of H4 proliferation, we examined early biochemical events in insulin signal transduction. Insulin treatment of serum-starved H4 rat hepatoma cells results in the activation of cytosolic S6 protein kinases; assays of H4 cytosolic extracts showed a progressive increase in total S6 kinase to a plateau at 10 min that was sustained thereafter for at least 1 hour

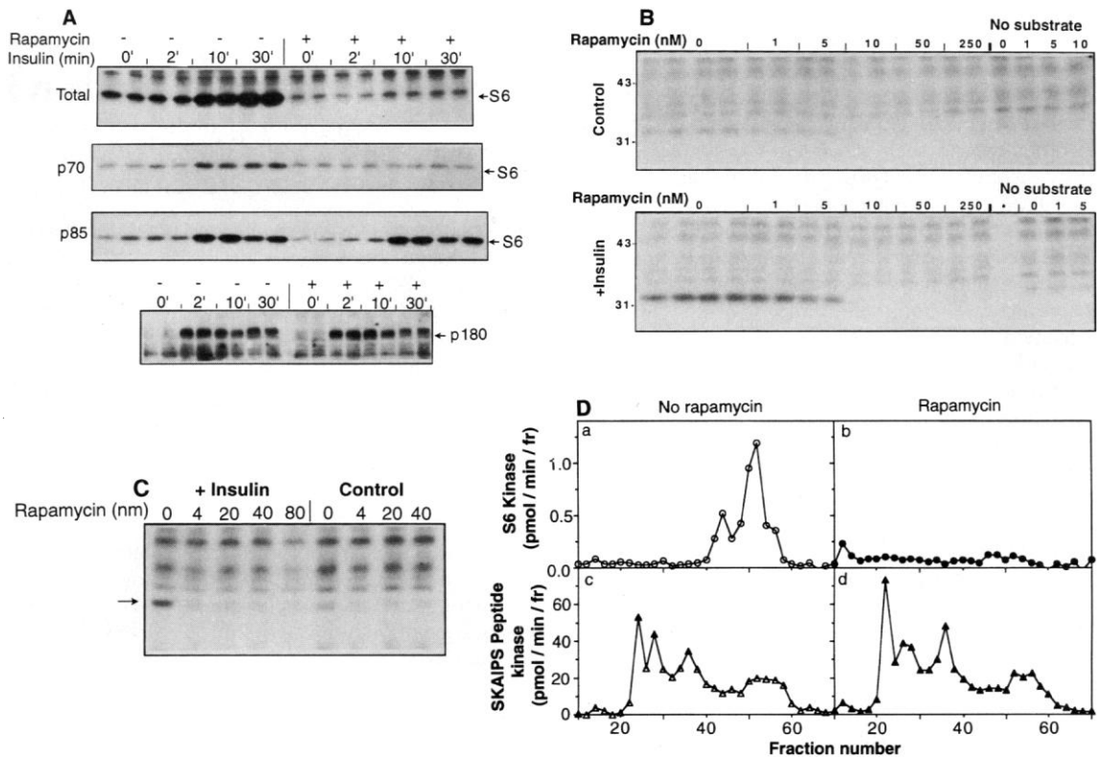

Fig. 2. Effect of rapamycin on insulin-regulated protein kinases in H4 hepatoma cells. (**A**) Serum-starved (24 hours) H4 cells were incubated in the presence or the absence of 20 nM rapamycin for 30 min before the addition of insulin (10^{-6} M). At the indicated times, cytosolic extracts were prepared. Aliquots of total extract (*20*) (top panel), extracts immunoprecipitated with affinity-purified polyclonal antibody to a peptide derived from p70 S6 kinase (*14*) (second panel), or p85 Rsk S6 kinase (third panel), were assayed for S6 kinase activity with 40*S* ribosomes as substrate. Bottom panel, proteins from H4 cell extracts Western-blotted with an antibody to phosphotyrosine. The region containing IRS-1 (P180) is shown. (**B**) Rapamycin-mediated inhibition of basal and insulin-stimulated S6 kinase activity. Rapamycin (0 to 250 nm) was added to serum-starved H4 cells 1 hour before harvest. Thirty minutes before harvest, the cells were either treated with insulin (10^{-6} M) or left untreated. Cells were harvested (*20*), and the kinase activity of cytosolic extracts detected as phosphorylation of 40*S* ribosomes (*7, 12*). (**C**) Effect of rapamycin on incorporation of ^{32}P into ribosomal protein S6 in intact H4 cells. Serum-starved (24 hours) H4 cells were incubated with serum-free medium containing 0.5 mCi of $^{32}P_i$ per 10-cm plate; after 1 hour, rapamycin (0 to 80 nm) was added. One hour later, insulin (10^{-6} M) was added to half of the plates. The cells were harvested 30 min later in extraction buffer (*20*) without Triton X-100, with aprotinin (10 U/ml) (0.5 ml per plate), and homogenized and centrifuged (10 min, 1000*g*). After further centrifugation (1.5 hours, 2 × 10^5*g*), sedimented material was subjected to SDS-PAGE. ^{32}P-labeled S6 is indicated by the arrow. (**D**) MonoQ chromatography of extracts from insulin-treated H4 cells. Serum-starved (24 hours) H4 cells were incubated in the absence (a and c) or the presence (b and d) of rapamycin (20 nM). After 30 min, insulin (10^{-6} M) was added to all plates. Cells were harvested 30 min thereafter into extraction buffer (*20*) without Triton X-100. Cytosolic extracts were chromatographed on a column (*23*). Fractions (fr) (1 ml) were assayed for kinase activities toward 40*S* ribosomes (a and b) and SKAIPS peptide (c and d) (*4, 7*).

(Fig. 2A) (*12*). Incubation of H4 cells with rapamycin for 1 hour before addition of insulin led to a dose-dependent inhibition of both basal and insulin-stimulated S6 kinase activity in the cytosolic extract that was essentially complete at 10 nM rapamycin (Fig. 2B). The rapamycin inhibition of cytosolic S6 kinase activity of H4 cells was accompanied by an inhibition of basal and insulin-stimulated phosphorylation of the ribosomal protein S6 in situ (Fig. 2C).

To determine whether the inhibition of total S6 kinase activity by rapamycin was a consequence of the inhibition of p70 or p85 Rsk S6 kinase, or both, we resolved cytosolic extracts by anion-exchange chromatography (Figs. 2D and 3A) and by immunoprecipitation (Fig. 2A). H4 cytosolic extracts contain a dominant peak of S6 kinase activity that was eluted from a MonoQ anion-exchange column near 0.25 M NaCl (Fig. 2D). This peak corresponds to the p70 S6 kinase (*13*), and recombinant p70 S6 kinase expressed in COS cells exhibits similar elution (*14*). This peak of S6 kinase activity was completely inhibited by rapamycin pretreatment (Fig. 2D). Rapamycin also caused loss of S6 kinase activity in immunoprecipitates prepared with antibodies to a peptide from the p70 S6 kinase (Fig. 2A). The p85 Rsk S6 kinase, which elutes from MonoQ between 0.05 and 0.1 M NaCl (Fig. 3A), contributes less than 5% of the total cytosolic S6 kinase activity in H4 cells (Fig. 2D). We examined the activity of p85 S6 kinase by immunoprecipitation with an antibody to a peptide from the p85 Rsk kinase protein; p85 Rsk S6 kinase underwent activation in response to insulin, peaking in activity at 10 min (Fig. 2A). Concentrations of rapamycin that caused maximal inhibition of mitogenesis (Fig. 1) and total inhibition of p70 S6 kinase activity (Fig. 2B) did not alter the time course or magnitude of the insulin activation of p85 Rsk S6 kinase (Fig. 2A).

We further evaluated the differential sensitivity of the p70 and p85 Rsk S6 kinases to inhibition by rapamycin by directly examining the activity of the recombinant S6 kinase expressed transiently in COS cells. COS cells endogenously expressed both p85 and p70 S6 kinase activities, which were separable by MonoQ anion-exchange chromatography and independently regulated (Fig. 3A). Active phorbol esters (Fig. 3A), serum, or epidermal growth factor (EGF), but not insulin, each stimulated the activity associated with the p85 Rsk kinase, but did not alter the activity of the p70 S6 kinase (*15*). The endogenous MAP kinase activity in COS cells was increased by the same stimuli that increased p85 Rsk S6 kinase activity (*15*). Recombinant epitope-tagged (r-epi) (*16*) versions of p70 S6 kinase, p85 Rsk S6 kinase, and rat p44 MAP kinase

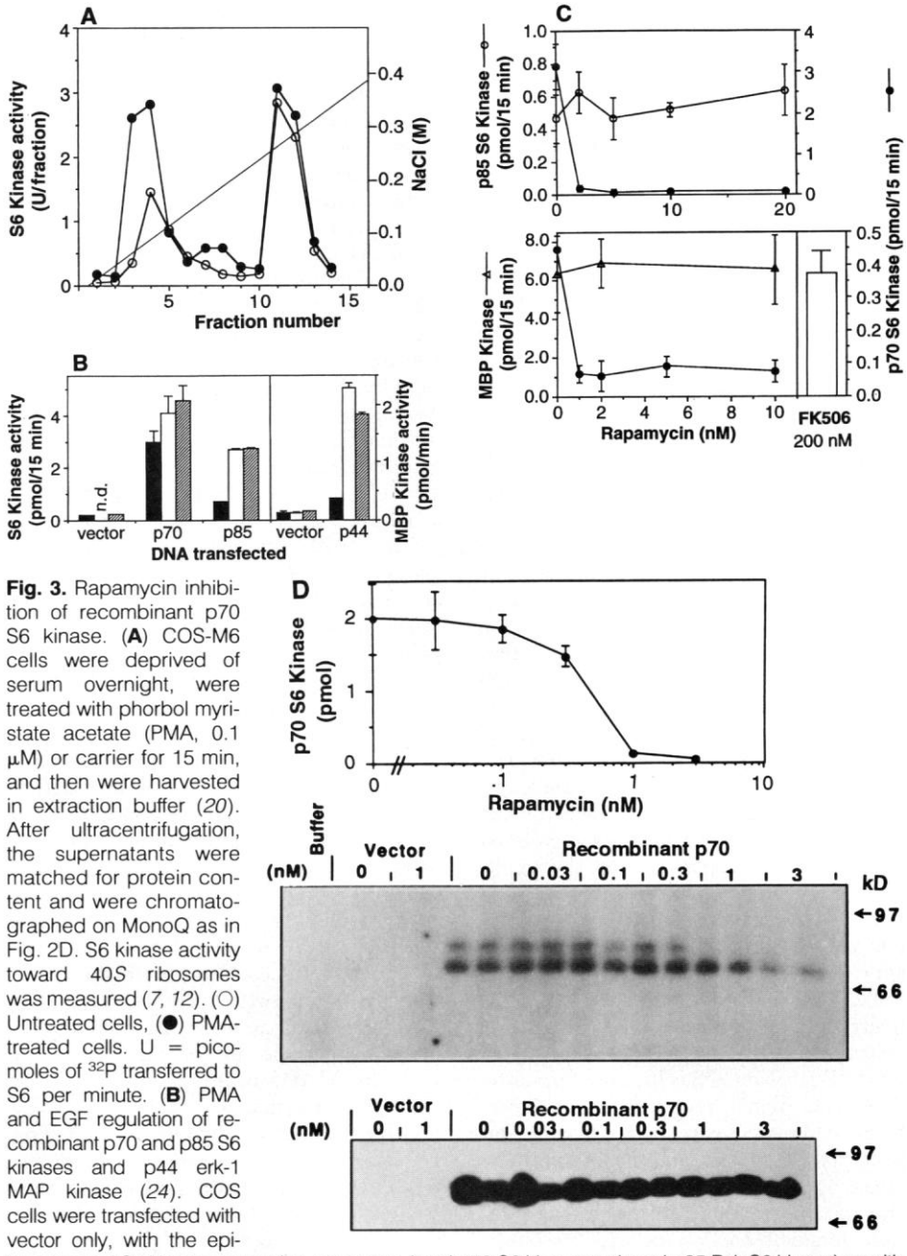

Fig. 3. Rapamycin inhibition of recombinant p70 S6 kinase. (**A**) COS-M6 cells were deprived of serum overnight, were treated with phorbol myristate acetate (PMA, 0.1 μM) or carrier for 15 min, and then were harvested in extraction buffer (*20*). After ultracentrifugation, the supernatants were matched for protein content and were chromatographed on MonoQ as in Fig. 2D. S6 kinase activity toward 40*S* ribosomes was measured (*7, 12*). (○) Untreated cells, (●) PMA-treated cells. U = picomoles of ^{32}P transferred to S6 per minute. (**B**) PMA and EGF regulation of recombinant p70 and p85 S6 kinases and p44 erk-1 MAP kinase (*24*). COS cells were transfected with vector only, with the epitope-tagged S6 kinase expression constructs (r-epi p70 S6 kinase and r-epi p85 Rsk S6 kinase) or with the epitope-tagged p44 MAP kinase (r-epi erk-1) (*24*). After 24 hours, cells were left untreated (solid bars) or treated with PMA (100 nM) for 15 min (open bars) or EGF (60 ng/ml) for 10 min (hatched bars), then were homogenized in extraction buffer (*20*). Recombinant proteins were immunoprecipitated by incubation with the monoclonal antibody 12CA5 (*22*), and S6 kinase activity in the washed immunoprecipitates was determined (*7, 12*). The r-epi erk-1 activity was assayed similarly with myelin basic protein (MBP) as substrate. Error bars indicate standard deviation ($n = 3$). (**C**) Recombinant p70 S6 kinase but not p85 S6 kinase or erk-1 is inhibited by rapamycin but not FK506. COS cells were transfected with r-epi p70 and r-epi p85 Rsk S6 kinase cDNAs (top panel), or r-epi p70 S6 kinase and r-epi erk-1 cDNAs (bottom panel); 48 hours later, the indicated concentrations of rapamycin were added to each plate 15 min before harvest. Recombinant proteins were immunoprecipitated and assayed for kinase activities as in Fig. 3B. Error bars indicate standard deviation (upper panel, $n = 5$; lower panel, $n = 6$). (**D**) Dose-response of rapamycin inhibition of r-epi p70 S6 kinase activity toward 40*S* subunits (top panel) (mean ± SD, $n = 6$) compared with r-epi p70 S6 kinase autophosphorylation (middle). COS cells were transfected with r-epi p70 S6 kinase cDNA. After 48 hours, rapamycin was added at the indicated concentrations. PMA (0.1 μM) was added 45 min later, and 15 min thereafter cells were extracted and were subjected to immunoprecipitation as in Fig. 3A. Autophosphorylation (middle panel) was measured by omitting the 40*S* subunits in the S6 kinase assay. The autoradiograph (middle) exhibits the ^{32}P incorporation into r-epi p70 polypeptide during a 15-min incubation with [γ-^{32}P]-labeled adenosine triphosphate. Portions of each immunoprecipitation were subjected to SDS-PAGE, were blotted onto PVDF membranes, and were probed with anti-p70 S6 kinase peptide antibody (*14*) (lower panel).

(erk-1), expressed transiently in COS cells, exhibited similar regulatory behavior. The r-epi p85 Rsk S6 kinase and r-epi p44 MAP kinase were activated by treatment of cells with phorbol 12-myristate 13-acetate (PMA) or EGF, whereas the recombinant p70 S6 kinase was constitutively active, and the activity was not altered significantly by PMA, EGF (Fig. 3B), or withdrawal or readdition of serum (*15*). Treatment of COS cells with rapamycin abolished the activity of r-epi p70 S6 kinase, whereas the basal (*15*) or PMA-stimulated activities of the r-epi p85 S6 kinase and an r-epi p44 MAP kinase were not affected by 10- to 20-fold higher concentrations of rapamycin (Fig. 3C). Rapamycin treatment did not inhibit the expression or recovery of the r-epi p70 S6 kinase polypeptide (Fig. 3D). Moreover, the inhibition of p70 S6 kinase activity was specific for rapamycin; treatment of cells with a 100-fold higher concentration of FK506 failed to inhibit r-epi p70 S6 kinase activity (Fig. 3C).

The p70 S6 kinase is activated by phosphorylation at multiple Ser and Thr residues (*13*). Both the endogenous and recombinant p70 S6 kinase polypeptide expressed in COS cells appear as a ladder of polypeptide bands after SDS–polyacrylamide gel electrophoresis (PAGE), of which only those with the slowest mobility coelute on MonoQ chromatography with the active enzyme (*14*). The slowed mobility on SDS-PAGE is abolished by treatment with protein phosphatase and reflects a phosphorylation-induced conformational change associated with the active state. Although virtually all recombinant p70 S6 kinase polypeptides contain ^{32}P when isolated from ^{32}P-labeled COS cells, only a minority show the highly retarded migration on SDS-PAGE seen with purified active rat liver p70 S6 kinase, and coelute with S6 kinase activity. After an in vitro autophosphorylation reaction, this electrophoretically retarded fraction of p70 S6 kinase polypeptides appears as a highly phosphorylated species migrating more slowly than the bulk of p70 S6 kinase; the latter shows much lower incorporation of ^{32}P relative to its abundance (Fig. 3D). The inhibition of the p70 S6 kinase activity seen at increasing concentrations of rapamycin was correlated with the disappearance of the slowly migrating ^{32}P-labeled band observed after the autophosphorylation reaction in vitro. By contrast, the in vitro autophosphorylation associated with the faster migrating p70 polypeptide (Fig. 3D) and the overall ^{32}P incorporation occurring in situ into the faster migrating p70 polypeptides, which are catalytically inactive toward 40S subunits, were only diminished at much higher concentrations of rapamycin (*15*). Thus, rapamycin inhibition of p70 S6 kinase activity toward S6 in 40S subunits is paralleled by the selective loss of autophosphorylating activity associated with the fully active (slowest migrating) p70 S6 kinase species.

This result could be explained if rapamycin, alone or as a complex with an FKBP, bound to and inhibited only the active conformation of p70 S6 kinase or prevented the accumulation of the active conformation of p70 S6 kinase by preventing its activation or accelerating its dephosphorylation. The effect of rapamycin, alone and as a complex with FKBP12 (*17*), an FKBP known to bind rapamycin with high affinity (*8*), on the activity of purified, fully active rat liver p70 S6 kinase (*4, 14*) was examined in vitro. Rapamycin (40 nM), added either alone or after prebinding to an equimolar concentration of recombinant FKBP12, did not alter p70 S6 kinase activity, or the rate at which rat liver p70 S6 kinase was inactivated by phosphatase-2A (*15*). The lack of direct inhibition of active p70 S6 kinase by rapamycin and FKBP12 suggests either that another FKBP is required for rapamycin activity or that rapamycin inhibits p70 S6 kinase indirectly, by inhibiting an upstream activator that is also crucial for mitogenesis.

The mechanism by which insulin mediates activation of cytosolic p70 S6 kinase is incompletely understood. Intrinsic tyrosine kinase activity of the insulin receptor results in tyrosine phosphorylation of a 180-kD polypeptide substrate termed IRS-1 (*16*). Insulin-stimulated tyrosine phosphorylation of IRS-1 in H4 cells was not altered by concentrations of rapamycin that abolished p70 S6 kinase activity (Fig. 2B). Insulin activates an array of proline-directed protein kinases in H4 hepatoma cells, including MAP kinases and a form of cdc2 that phosphorylate a putative regulatory domain on intact p70 S6 kinase (*3, 7*). The activation of these enzymes can be monitored with a synthetic polypeptide substrate, SKAIPS peptide, corresponding to these Ser Pro/Thr Pro-rich p70 S6 kinase regulatory sequences (*7*). Rapamycin, at concentrations that completely inhibit activation of p70 S6 kinase, did not alter the activation of these SKAIPS peptide kinases in response to insulin (Fig. 2D), indicating that MAP kinase and cdc2 activities are unaffected by drug (*18*). Thus, the target for rapamycin appears to be situated downstream of the insulin receptor kinase and tyrosine phosphorylation of IRS-1 on a signal transduction pathway distinct from that mediating activation of the erk-1/erk-2 MAP kinases. In the interleukin-2–dependent CTLL-20 cell line, interleukin-2 stimulates, and rapamycin inhibits, S6 kinase activity, whereas the PMA-stimulated activities of p85 Rsk S6 kinases and p42 MAP kinases are not inhibited by rapamycin (*19*). Thus, the mechanism of rapamycin action in lymphoid cells is also likely to involve a ubiquitous signal transduction element shared with nonlymphoid lineages. The rapamycin target might be a proximate upstream activator of the p70 S6 kinase, such as an activating p70 S6 kinase-kinase or a regulator of such an enzyme, and appears to be a crucial element linking growth factor receptors to subsequent intracellular processes regulating proliferation.

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20. H4 hepatoma and COS cells were harvested by homogenization in an extraction buffer containing 10 mM potassium phosphate (pH 6.5), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), 1 mM vanadate, 50 mM β-glycerophosphate, Triton X-100 (0.1%), 2 μM leupeptin, 2 μM pepstatin, 0.2 mM phenylmethylsulfonyl fluoride (PMSF). Extracts were centrifuged for 1.5 hours at 2 × $10^5 g$ and matched for protein content before chromatography or immunoprecipitation. Immune complexes bound to protein A Sepharose were washed three times in extraction buffer containing 0.25 M NaCl, and once in 20 mM

Tris Hcl (pH 7.4), 1 mM EGTA, 2 mM EDTA, 2 mM DTT, 10 mM β-glycerophosphate, Triton X-100 (0.1%), glycerol (10%), before assay of S6 kinase activity.
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23. Cystolic extracts were matched for protein content, diluted with three parts of chromatography buffer [50 mM, β-glycerophosphate (pH 7.2), 1 mM DTT, 1 mM EGTA, 0.1 mM vanadate and applied to a MonoQ HR (515) column]. The column was eluted with a 90-ml gradient of NaCl (0 through 0.4 M final) in chromatography buffer.
24. The cDNA encoding rat p70 S6 kinase αI (*14*), rat p85 S6 kinase (*3*), and rat erk-1 (*21*) were modified by insertion of a nine amino acid–peptide epitope derived from influenza virus hemagglutinin (*22*) at the NH_2-terminus of each polypeptide, immediately after the initiator methionine.
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A Point Mutation of the α_2-Adrenoceptor That Blocks Coupling to Potassium But Not Calcium Currents

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The α_{2A}-adrenergic receptor (adrenoceptor) was stably expressed in AtT20 mouse pituitary tumor cells; adrenoceptor agonists inhibited adenylyl cyclase, inhibited voltage-dependent calcium currents, and increased inwardly rectifying potassium currents. An aspartic acid residue (Asp^{79}) highly conserved among guanine nucleotide–binding protein (G protein)–coupled receptors was mutated to asparagine; in cells transfected with the mutant α_2-receptor, agonists inhibited adenylyl cyclase and calcium currents but did not increase potassium currents. Because distinct G proteins appear to couple adrenoceptors to potassium and calcium currents, the present findings suggest that the mutant α_2-adrenoceptor cannot achieve the conformation necessary to activate G proteins that mediate potassium channel activation.

The α_2-adrenergic receptors (α_2ARs) belong to the superfamily of G protein–coupled receptors, specifically to the branch of this family consisting of receptors coupled to the inhibition of adenylyl cyclase, the inhibition of Ca^{2+} currents, and the activation of K^+ currents by pertussis toxin (PTX)–sensitive G proteins (*1*). One or both of these latter two effects is responsible for the immediate inhibition of neurotransmitter release and neuronal firing produced by activation of presynaptic and postsynaptic α_2ARs on mammalian neurons (*1*). Multiple α_2AR subtypes have been identified by both pharmacological and molecular biological approaches, and site-directed mutagenesis of α_2ARs has identified several amino acids in transmembrane regions II through V as sites of interaction for agonist binding and for receptor coupling to the inhibition of adenylyl cyclase (*1, 2*). However, it is not known whether cloned α_2ARs, when expressed in a heterologous system, can couple to diverse ion channels or whether specific domains can be identified that participate in coupling to specific diverse effector systems. We investigated the coupling of a stably transfected α_{2A}AR (*3*) to K^+ currents, Ca^{2+} currents, and adenylyl cyclase and the consequences of a single amino acid mutation [converting aspartic acid to asparagine at position 79 (Asn^{79} α_2AR)] on the coupling to these three effector systems.

The AtT20 cell does not express endogenous α_2ARs (Table 1) but contains somatostatin receptors that couple to inhibition of adenylyl cyclase (*4*), inhibition of Ca^{2+} currents (*5*), and activation of an inwardly rectifying K^+ current (*6*). Thus, we used AtT20 cells to evaluate the functional properties of wild-type (WT) α_2ARs or Asn^{79} α_2ARs. We compared somatostatin-induced alterations in K^+ and Ca^{2+} currents (*7*) with responses to the α_2AR agonists clonidine and UK 14304 in permanent transformants of AtT20 cells expressing recombinant WT or Asn^{79} α_2ARs (*8*).

The α_2AR agonist UK 14304 increased the K^+ current in cells expressing the WT α_2AR (Fig. 1); 95% of the current induced by UK 14304 was blocked by 1 mM Ba^{2+} (Fig. 1A), as would be expected if the agonist were opening inwardly rectifying K^+ channels (*9*). Somatostatin (100 nM) increased this current by two- to tenfold in all mock-transfected cells and in cells transfected with the WT α_2AR or Asn^{79} α_2AR (Fig. 1, B through E). Maximally effective concentrations of UK 14304 or clonidine produced a 1.5- to 8-fold increase in K^+ current in cells expressing the WT α_2AR (Fig. 1, B through D). Concentrations of clonidine and UK 14304 that produced half-maximal activation (EC_{50}) of the K^+ current were 14 and 30 nM, respectively, in cells expressing the WT α_2AR (Fig. 2A); these are similar to the EC_{50} values for the inwardly rectifying K^+ conductance activated by pharmacologically characterized α_{2A}ARs in autonomic enteric and central locus coeruleus neurons (*10*). The actions of maximally effective concentrations of somatostatin and AR agonists were not additive ($n = 22$), which is evidence that the transfected WT α_2AR couples to the same set of K^+ channels as does the endogenous somatostatin receptor. In contrast to the WT α_2AR, the mutant Asn^{79} α_2AR did not activate K^+ currents (Fig. 1, B, C, and E), even in the presence of 10,000-fold higher concentrations of clonidine or UK 14304 (Fig. 2A).

In contrast to their effects on K^+ currents, α_2AR agonists were effective in inhibiting Ca^{2+} currents in AtT20 cells expressing WT α_2ARs or Asn^{79} α_2ARs (Fig. 3). In either case, the inhibition of Ca^{2+} currents by AR agonists was not quantitatively different from the inhibition of Ca^{2+} currents by somatostatin acting at endogenous receptors (Fig. 3, A and B). Somatostatin inhibits two high-voltage–activated (HVA) Ca^{2+} currents in AtT20 cells, a dihydropyridine-sensitive (HVA/L-type) current and a dihydropyridine-insensitive (HVA/N-type) current (*5*); α_2AR agonists similarly inhibited HVA/L- and HVA/N-type Ca^{2+} currents in cells expressing either WT or Asn^{79} α_2ARs (Fig. 3, C and D). Somatostatin inhibited the Ca^{2+} current in 92% of mock-transfected cells examined but inhibited Ca^{2+} currents in only 50% of cells expressing WT α_2ARs or Asn^{79} α_2ARs (Fig. 3B). The percentage of cells in which somatostatin inhibited the Ca^{2+} current was not correlated with the cell cycle nor the time after cell passage. The explanation for this observation is unclear because the percentage of cells responding to somatostatin with an increase in K^+ current was similar in all cells (Fig. 1C).

There were no apparent differences in the concentration-response curves for clonidine-induced inhibition of the Ca^{2+} current in cells expressing WT or Asn^{79} α_2ARs (Fig. 2B) or the norepinephrine-mediated responses (measured in the presence of pro-

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