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Role of βγ Subunits of G Proteins in Targeting the β-Adrenergic Receptor Kinase to Membrane-Bound Receptors

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The rate and extent of the agonist-dependent phosphorylation of β_2-adrenergic receptors and rhodopsin by β-adrenergic receptor kinase (βARK) are markedly enhanced on addition of G protein βγ subunits. With a model peptide substrate it was demonstrated that direct activation of the kinase could not account for this effect. G protein βγ subunits were shown to interact directly with the COOH-terminal region of βARK, and formation of this βARK-βγ complex resulted in receptor-facilitated membrane localization of the enzyme. The βγ subunits of transducin were less effective at both enhancing the rate of receptor phosphorylation and binding to the COOH-terminus of βARK, suggesting that the enzyme preferentially binds specific βγ complexes. The βγ-mediated membrane localization of βARK serves to intimately link receptor activation to βARK-mediated desensitization.

The efficacy with which receptors coupled to G proteins mediate stimulation in response to extracellular signals is modulated by dynamic processes. In most systems, persistent stimulation is followed by diminished responsiveness, a phenomenon generally termed desensitization. Receptor phosphorylation is one mechanism whereby receptor function may be regulated (*1*). In particular, agonist-dependent phosphorylation of G protein–coupled receptors is thought to participate in agonist-specific or homologous desensitization (*2*). The two systems in which this process has been most extensively characterized are β_2-adrenergic receptor (β_2AR) phosphorylation by βARK (*3*) and rhodopsin phosphorylation by rhodopsin kinase (RK) (*4*). In each case receptor phosphorylation is stimulus-dependent and contributes to receptor desensitization (*4*, *5*). βARK phosphorylates a number of other G protein–coupled receptors, including purified reconstituted α_2-adrenergic (*6*), muscarinic cholinergic (*7*), and rhodopsin (*8*) receptors in an agonist-dependent manner. Similarly, RK phosphorylates agonist-occupied β_2AR (*8*). Both βARK and RK are cytosolic enzymes that rapidly translocate to the plasma membrane on receptor stimulation (*9*); however, the molecular mechanisms underlying this process have remained obscure.

The βγ subunits of G proteins have recently been shown to activate a partially purified enzyme preparation that phosphorylates muscarinic cholinergic receptors and has properties similar to those of βARK (*10*). We now demonstrate that βγ subunits interact directly with recombinant βARK-1 and that this interaction serves to target the enzyme to membrane-bound receptors.

The agonist-dependent phosphorylation of reconstituted purified β_2AR by a purified preparation of βARK was enhanced when phosphorylation occurred in the presence of βγ subunits purified from bovine brain. Addition of βγ subunits enhanced both the initial rate and the maximal extent of phosphorylation (Fig. 1). At a molar ratio of βγ:βARK of 10:1, both the initial rate and the maximal extent of phosphorylation of agonist-occupied β_2AR were about 13

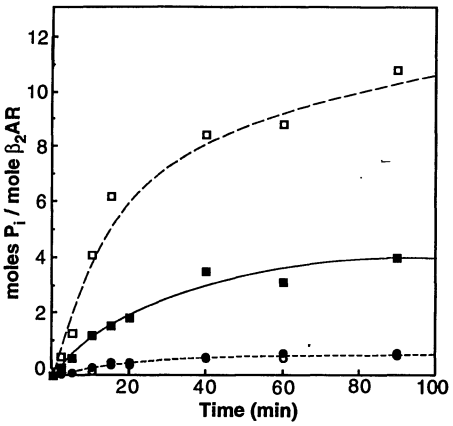

Fig. 1. Enhancement of βARK-mediated β_2AR phosphorylation by bovine brain βγ. Human β_2AR and bovine βARK were expressed in Sf9 cells and were purified with modifications of published procedures (*11*, *12*). The partially purified receptor was reconstituted into phospholipid vesicles (*15*, *16*) and was phosphorylated. Reconstituted β_2AR (20 nM) was incubated with βARK (30 nM) in 20 mM tris-HCl (pH 8.0), 2 mM EDTA, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) (buffer A) containing 100 μM ATP (~3000 cpm/pmol) and (−)isoproterenol (100 μM) in a total volume of 25 μl (○). Alternatively, 150 nM βγ (molar ratio βγ:βARK, 5:1) (■), 300 nM βγ (molar ratio βγ:βARK, 10:1) (□), or 150 nM βγ, 1.5 μM α_{i1} (molar ratio βγ:βARK:α_{i1}, 5:1:50) (●) was included in the phosphorylation incubation. βγ was purified from bovine brain as described in (*14*). At the times indicated, we stopped reactions by the addition of an equal volume of SDS sample-loading buffer. We subjected samples to electrophoresis on SDS-polyacrylamide gels and determined phosphorylation stoichiometries by excising and by counting the bands corresponding to the phosphorylated β_2AR receptor. We determined phosphorylation stoichiometries on the assumption that all reconstituted receptor was accessible to kinase.

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times those in the absence of βγ. Enhancement of phosphorylation appeared to be dependent on the presence of uncomplexed βγ subunits, and the addition of βγ subunits together with the free α subunit of the G protein G_{i1} (molar ratio α_{i1}:βγ, 10:1) negated the effect of free βγ subunits alone (Fig. 1) (*11–14*).

The initial rates of phosphorylation of both β_2AR (Fig. 2A) (*15, 16*) and rhodopsin (Fig. 2B) by βARK were enhanced in a dose-dependent fashion on addition of βγ subunits. The concentration of βγ subunits giving half-maximal activation of βARK activity, the apparent Michaelis constant (K_m) for βγ, was ~50 nM (a 1.7:1 molar ratio of βγ:βARK) for β_2AR phosphorylation. Similarly, for rhodopsin phosphorylation the apparent K_m for βγ was ~30 nM (a 1:1 molar ratio of βγ:βARK). Addition of βγ subunits also enhanced the rate and extent of β_2AR phosphorylation in the presence of an antagonist (propranolol). However, at any particular concentration of βγ at least a sevenfold enhancement of initial rate of phosphorylation was observed in the presence of an agonist. Thus, even in the presence of βγ subunits, β_2AR phosphorylation by βARK is enhanced on agonist occupancy of the receptor. The βγ subunits from a bovine retinal G protein, transducin, also enhanced βARK activity, but transducin βγ was approximately 10- and 90-fold less potent than bovine brain βγ in the β_2AR and rhodopsin systems, respectively. The maximal extent of phosphorylation of both agonist-occupied β_2AR (~11 moles of P_i per mole of β_2AR) and light-activated rhodopsin (~7 moles of P_i per mole of rhodopsin) was, however, similar in the presence of βγ from either bovine brain or transducin.

To determine if direct activation of βARK activity accounted for the enhanced rate of receptor phosphorylation observed on addition of βγ subunits, we studied phosphorylation of a model peptide substrate in the presence of a saturating concentration of adenosine triphosphate (ATP). The rate of peptide phosphorylation was independent of the addition of βγ subunits over a range of peptide concentrations (0.05 to 5.0 mM) (Fig. 3) (*17*). A slight enhancement (<2-fold) of βARK activity is observed against the peptide substrate on addition of phospholipid vesicles (*18*). Because this in no way accounts for the enhancement (>10-fold) of receptor phosphorylation observed on addition of βγ subunits, it is necessary to invoke a mechanism other than direct enzyme activation to explain this effect.

Half-maximal activation of βARK activity was observed in the presence of approximately equimolar ratios of βγ:βARK, suggesting that a specific physical association between these components may function in enhancing the rate of receptor phosphorylation without directly activating enzyme activity. This idea was supported by the observation that both the rate and extent of β_2AR phosphorylation by either adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) or protein kinase C (PKC) were unaffected by addition of bovine brain βγ subunits (*18*). Thus, activation of receptor phosphorylation by βγ subunits appears to be a phenomenon specific to βARK.

To test the notion that there is a specific physical interaction between βARK and βγ, we made use of the fact that pertussis toxin–catalyzed adenosine diphosphate (ADP)–ribosylation of $G_{o\alpha}$ subunits is dependent on the presence of βγ subunits. Addition of βARK to such a system specifically inhibited the ADP-ribosylation of $G_{o\alpha}$, presumably by forming βARK-βγ complexes, which reduce the concentration of free βγ available for association with $G_{o\alpha}$ (Fig. 4A).

Despite the apparent functional and structural similarities between RK and βARK, βγ subunits did not alter the rate at which RK phosphorylated the β_2AR (*18*). The rate of RK phosphorylation of rhodopsin is actually inhibited in the presence of βγ subunits purified from either transducin or bovine brain G proteins (*10, 19*). Examination of the amino acid sequences of βARK and RK reveals a structural difference. As compared to RK, βARK has an extended COOH-terminus with an additional 125 amino acids (*20*). Because RK is insensitive to βγ, we examined the possibility that the extended COOH-terminus of βARK is the site of interaction with βγ. A fusion protein in which the COOH-terminal coding sequence of βARK was ligated in frame to the sequence encoding glutathione-S-transferase (GST) was expressed in *Escherichia coli* and purified. The fusion protein contained the COOH-terminal 222 amino acids of βARK (residues 467 to 689). The fusion protein when coupled to glutathione-S-Sepharose bound βγ subunits from bovine brain (Fig. 4B) (*21*). GST itself did not bind βγ subunits, indicating that the βγ binding capacity is contained within the βARK COOH-terminal amino

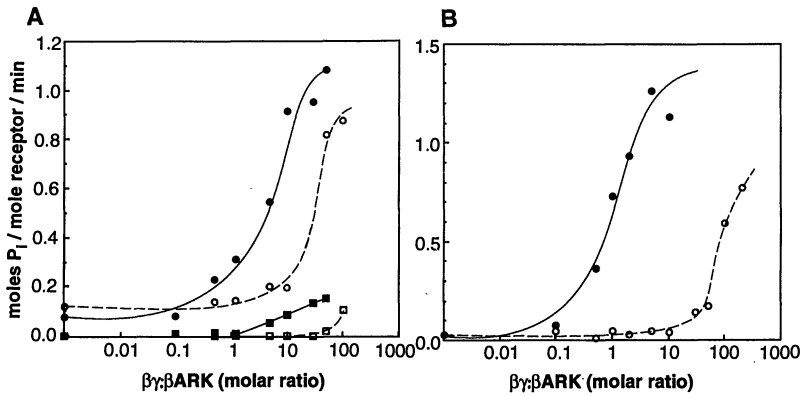

Fig. 2. Dose-dependent activation of βARK by βγ subunits. Reconstituted β_2AR in the presence of (**A**) (−)isoproterenol (100 μM) or (**B**) light-activated rhodopsin (each at a final concentration of 20 nM) were phosphorylated as described (Fig. 1) in the presence of various concentrations of βγ subunits purified from bovine brain ($\beta\gamma_B$, ●) or from bovine retina ($\beta\gamma_T$, ○). Rhodopsin and the βγ subunits of transducin were purified from bovine rod outer segments as described (*15, 16*). Reconstitution of the purified rhodopsin was performed as described (*13*). β_2AR was also phosphorylated in the presence of 30 μM propranolol and $\beta\gamma_B$ (■) or $\beta\gamma_T$ (□).

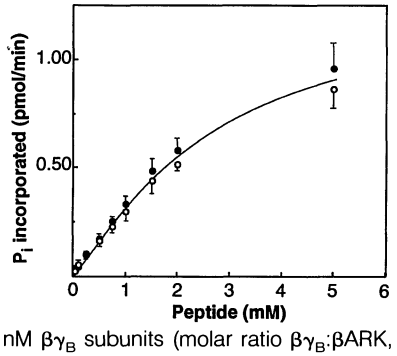

Fig. 3. βARK phosphorylation of peptide substrates in the presence of $\beta\gamma_B$ subunits. The synthetic peptide RRREEEEESAAA, at the concentrations indicated, was incubated with purified βARK (25 nM) in 20 mM tris-HCl, (pH 8.0), 2 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 100 μM γ-^{32}P–labeled ATP (250 to 500 cpm/pmol), 10 μM protein kinase inhibitor (PKI), and bovine serum albumin (BSA) (0.5 mg/ml), in a final volume of 25 μl at 30°C for 10 min. We terminated the reaction by the technique of adsorption onto P-81 paper as described (*17*). Activity of βARK was defined as the difference in phosphate incorporation in the presence and absence of peptide. Phosphorylation reactions were performed in the absence (○) or presence of 255 nM $\beta\gamma_B$ subunits (molar ratio $\beta\gamma_B$:βARK, 10:1) (●). The results are the mean ± SEM for three separate experiments.

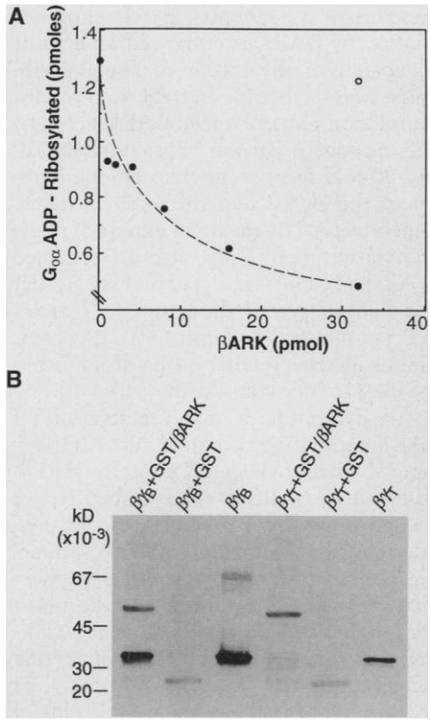

Fig. 4. Physical association of βARK and βγ. (**A**) Inhibition of pertussis toxin catalyzed ADP-ribosylation of $G_{o\alpha}$ by βARK. Purified $G_{o\alpha}$ (375 nM) and βγ (6.25 nM) were mixed with βARK at the concentrations indicated in the figure. A control point with heat-inactivated βARK (○) is also shown. ADP-ribosylation reactions were performed in a total volume of 40 μl, containing 75 mM tris-HCl, (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 100 μM GDP, 2 mM DTT, 2.5 μM [^{32}P]-labeled nicotinamide adenine dinucleotide (NAD) (~10,000 cpm/pmol), 0.5 mM dimyristoylphosphatidylcholine and pertussis toxin (5 mg/ml), as described (*14*). (**B**) Interaction of βγ subunits and the COOH-terminal 222 amino acids of βARK. Bovine brain βγ ($\beta\gamma_B$, 38 nM) or transducin βγ ($\beta\gamma_T$, 154 nM) was incubated in a total volume of 65 μl, containing phosphate-buffered saline (PBS) and lubrol (0.01%), with either GST (1.5 μM) or a GST-βARK fusion protein (550 nM) for 20 min at 4°C. The fusion protein, which contained the COOH-terminal 222 amino acid residues of βARK ligated to sequences encoding GST, was expressed in *E. coli* and was purified as described (*21*). After the initial incubation of βγ with GST or GST-βARK glutathione Sepharose (20 μl of a 50% slurry in PBS, Pharmacia Diagnostics, Silver Spring, Maryland) was added, and incubation was continued on ice for 20 min. The Sepharose beads containing bound GST or GST-βARK were subsequently washed three times with PBS (400 μl) containing lubrol (0.01%), subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (4 to 15% gradient gels), and transferred to nitrocellulose for protein immunoblot analysis. Antibodies to β and GST were used at dilutions of 1:1000 and 1:25,000, respectively. Blots were developed with goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase (Bio-Rad, Richmond, California), according to the manufacturer's instructions.

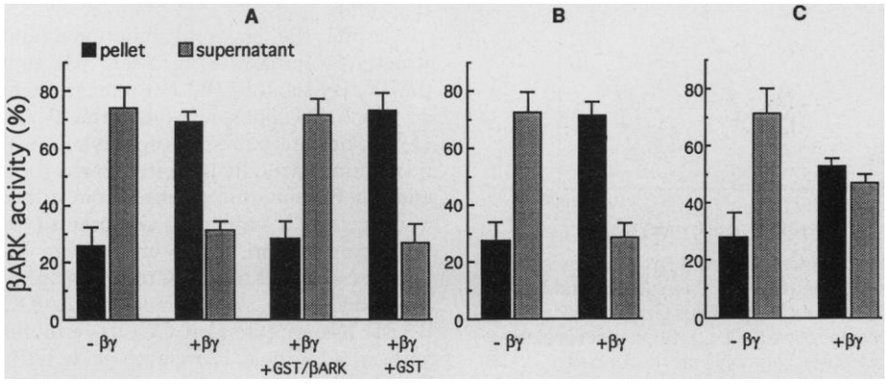

Fig. 5. Membrane targeting of the βARK/βγ complex. Urea-stripped ROS membranes were prepared (*27*). The membranes contained approximately 95% rhodopsin with negligible receptor kinase activity. Purified βARK (15 nM) was incubated with 10 μl of native (**A** and **B**) or heat-inactivated (**C**) ROS membranes (final concentration of rhodopsin ~5 μM) in buffer A in a final volume of 50 μl. Incubations were performed at 22°C for 3 min under bright illumination (A and C) or in the dark (B). βγ subunits purified from bovine brain (0.6 μM), GST-βARK fusion protein (3 μM), or GST (3 μM) were included in the incubations as indicated. Samples were subsequently centrifuged at 350,000*g* for 5 min. After centrifugation the supernatants were rapidly removed, were supplemented with ROS membranes (10 μl) and [γ-^{32}P]ATP (final concentration 100 μM, ~3000 cpm/pmol), and were incubated under illumination at 30°C for 5 min. The sedimented ROS membranes were resuspended in buffer A containing 100 μM [γ-^{32}P]ATP and were incubated under identical conditions. The βγ subunits (0.3 μM) were added to both the supernatant and sedimented fractions of samples that lacked βγ during the primary incubation. We terminated reactions by addition of an equal volume of SDS-PAGE sample buffer, and we fractionated proteins by SDS-PAGE. The bands corresponding to phosphorylated rhodopsin were excised and were counted. The total radioactivity incorporated into rhodopsin in both the supernatant and pellet fractions for a given sample was taken as 100%. The data shown are the means ± SEM for three separate experiments.

acid sequence. βγ subunits derived from transducin, which are ~30-fold less potent than βγ from bovine brain at activating βARK, did not bind to the βARK-GST fusion protein. These results demonstrate that there is a direct physical interaction between the COOH-terminal 222 amino acids of βARK and βγ subunits from bovine brain; moreover, this interaction appears to be specific.

The cDNAs encoding G protein γ subunits all encode polypeptides ending in a COOH-terminal CAAX sequence [a conserved Cys and then two aliphatic amino acids and a final residue of any amino acid (*22*)]. This sequence directs the covalent processing of these proteins, including the proteolytic removal of the last three COOH-terminal amino acids, carboxyl-methylation and lipidation. The γ subunit of transducin (γ_T) has a farnesyl isoprenoid (15-carbon) attached to the sulfydryl group of the Cys in the CAAX-motif (*23*), whereas γ subunits of brain G proteins (γ_2 and γ_3) are modified through the covalent attachment of a 20-carbon geranylgeranyl isoprenoid moiety (*24*). This isoprenylation of the γ subunits of G proteins is necessary for the membrane targeting of the βγ complex (*25*). RK, which is insensitive to βγ activation, is isoprenylated (*26*). We therefore investigated the possibility that βγ enhances the rate of phosphorylation of receptor substrates incorporated into phospholipid by promoting the membrane localization of βARK. Rod outer segment membranes were used for these experiments because they are a readily available source of highly concentrated rhodopsin, and βγ subunits enhance the rate at which βARK phosphorylates these receptors (Fig. 2).

In the absence of βγ subunits, βARK activity is largely (70%) in the soluble fraction (Fig. 5, A, B, and C) (*27*). On addition of βγ, this ratio was reversed with 70% of enzyme activity being associated with the particulate fraction containing the receptors and only 30% remaining in the soluble fraction (Fig. 5, A and B). Furthermore, addition of an excess of the GST-βARK fusion protein (molar ratio of fusion protein: βγ:βARK, 10:1:0.2) inhibited the translocation of βARK associated with βγ addition, presumably because the fusion protein binds βγ subunits (Fig. 4). GST, which does not bind βγ, had no effect on βγ-mediated βARK translocation (Fig. 5A).

Heat inactivation of rhodopsin, which prevents βARK-mediated phosphorylation (*28*), impaired translocation of the βARK-βγ complex (Fig. 5C). In the absence of active receptor, βγ addition led to only ~50% of the βARK being membrane associated. This indicates that, although association of βARK with isoprenylated βγ

contributes to βARK translocation, the receptor also participates in the membrane association of this enzyme. The enhanced rate of βARK-mediated receptor phosphorylation observed in the presence of βγ would thus appear to be a consequence of the membrane localization of the kinase through formation of a receptor–βARK-βγ ternary complex.

Unlike receptor phosphorylation, the receptor-facilitated membrane localization of the βARK-βγ complex was stimulus independent (Fig. 5, A and B). Thus, the βARK-βγ complex binds to either activated or inactivated receptor. In vivo, G proteins undergo a cycle of guanine nucleotide exchange during which they exist in two distinct states, an inactive conformation in which $G_{\alpha\text{-guanosine diphosphate}}$ ($G_{\alpha\text{-GDP}}$) is complexed to βγ, and an active state in which $G_{\alpha\text{-guanosine triphosphate}}$ ($G_{\alpha\text{-GTP}}$) subunits, capable of interacting with and activating various effectors (*29*), are dissociated from βγ. Because the exchange of GDP for GTP is stimulated by the ligand-activated receptor, uncomplexed βγ subunits should be available in vivo only in the presence of agonist-occupied receptor. Thus, in this sense, translocation of βARK through formation of the βARK-βγ–receptor complex is predicted to be agonist dependent in vivo. Indeed, addition of α subunits to βγ, a condition that favors formation of the heterotrimeric G protein, inhibited the enhancement of βARK-mediated receptor phosphorylation observed on addition of βγ alone.

RK, unlike βARK, ends with a CAAX sequence and is farnesylated and carboxylmethylated in vivo. Furthermore, the posttranslationally modified form of this enzyme is about four times as active in phosphorylating rhodopsin as its unfarnesylated counterpart (*26*). The enhanced rate of rhodopsin phosphorylation accompanying farnesylation is caused by light-dependent translocation of RK to rhodopsin-containing membranes (*30*). Both βARK and RK thus appear to translocate to membranes in a prenylation-dependent fashion.

βγ subunits have been implicated in regulation of a K^+ channel (*31*) and activation of phospholipase A2 (*32*). βγ also stimulates type II adenylyl cyclase in the presence of activated α_s, thus acting as a conditional activator of cAMP synthesis (*33*). The results presented in this study suggest another role of the βγ dimer, the enhancement of agonist-stimulated receptor phosphorylation and desensitization.

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Requirement for the Adenovirus Type 9 *E4* Region in Production of Mammary Tumors

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Oncogenic viruses demonstrating a strict tropism for the mammary gland provide special opportunities to study the susceptibility of this tissue to neoplasia. In rats, human adenovirus type 9 (Ad9) elicits mammary fibroadenomas that are similar to common breast tumors in women, as well as phyllodes-like tumors and mammary sarcomas. By constructing recombinant adenoviruses between Ad9 and Ad26 (a related nontumorigenic virus), it was shown that the Ad9 *E4* region was absolutely required to produce these mammary tumors. This indicates that an adenovirus gene located outside the classic transforming region (*E1*) can significantly influence the in vivo oncogenicity of an adenovirus. Consistent with a direct role in mammary gland oncogenesis, the Ad9 *E4* region also exhibited transforming properties in vitro. Therefore, the Ad9 *E4* region is a viral oncogene specifically involved in mammary gland tumorigenesis.

Human adenoviruses are classified as DNA tumor viruses because of their ability to induce tumors in rodents or to transform rodent cells in culture, and the E1 region (*E1A* and *E1B* genes) encodes the proteins responsible for the oncogenic properties of these viruses (*1*). The E1A proteins alone are capable of immortalizing primary rodent cells in culture (*2*) and, in cooperation with the E1B proteins (*3*), produce fully transformed cells. The transforming properties of these viral oncoproteins result, at least in part, from an ability to complex with important cellular proteins (*4–8*). Such complexes between viral oncoproteins and cellular proteins are believed to perturb the normal functions of the targeted host proteins, most of which appear to regulate important control points of the cell cycle.

Among the oncogenic adenoviruses, the subgroup D adenovirus Ad9 is unique because it elicits exclusively estrogen-depen-