Met is constitutively activated in those cells.

The coordination of differentiation and proliferation is an essential feature in the successful development or replacement of tissues, but it is not clear how the decline of proliferation is related to the onset of differentiation. The expression of Met in lumen border cells in vivo and in vitro and the colocalization of Met and anti–P-Tyr immunofluorescence suggest that the Met receptor may be involved in differentiation toward lumen formation. Spatial organization might be dictated by the restricted distribution of the receptor (Fig. 3) (*4*). It is possible that the motogenic (scattering) activity of HGF/SF (*28*) is a fundamental part of this formation.

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17. For immunofluorescence assays, T47D cells treated with Bouin's fixative, breast cancer biopsies, and mouse embryos were paraffin-embedded, serial-sectioned (*16*), and blocked for 10 min with immunostaining kit blocking reagents (Biomeda, Foster City, CA). Primary antibody {C28, rabbit antibody to human Met [1:100 dilution in phosphate-buffered saline (PBS) (*16*)]; C200, rabbit antibody to the extracellular domain of the Met peptide, amino acids 643 to 663 [M. Park *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6379 (1987)]; SP260, rabbit antibody to mouse Met (1:100 dilution in PBS) (*21*); or 4G10, mouse monoclonal antibody to P-Tyr [D. K. Morrison *et al.*, *Cell* **58**, 649 (1989)]} was added and incubated for 2 hours at room temperature. Secondary antibody incubations (donkey antibody to rabbit immunoglobulin coupled to phycoerythrin diluted 1:50 in PBS and donkey antibody to mouse immunoglobulin coupled to fluorescein isothiocyanate diluted 1:100 in PBS (Jackson ImmunoResearch Laboratories Westgrove, PA) were performed for 1 hour at room temperature. After an extensive washing, cells were fixed with gel mount (Biomeda). Fluorochrome-labeled cells were examined with a Zeiss CLSM with the following configuration: 25-mW Ar and He-Ne lasers with 488, 514, and 543 maximum lines and control Indec (Sungate, Capitola, CA) software for image acquisition of *x-y* scan and *z*-series scan three-dimensional visualization. Fluorescent and Nomarski [J., Padawer, *J. R. Microsc. Soc.* **88**, 305 (1968)] images were generated. Photographs were taken with a color video printer mavigraph (Sony) and UPC-5010a color print paper (Sony). When comparing the fluorescence intensity, we used identical parameters for each image (scanning line, laser light, contrast, and brightness) and assessed quantitation of the relative fluorescence by using Histogram, an Indec CLSM image processor option.
18. I. Tsarfaty, unpublished results.
19. Similar competition as in Fig. 5, A and B.
20. We observed intense staining in duct-forming cells as compared to adjacent tissues in all samples similar to the staining in T47D cells (Fig. 2, C through E).
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23. The quantitative determination of Met-specific immunofluorescence was performed as described in Fig. 2, C through E (*17*).
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25. Cells were grown on 16-chamber Labtek slides (Nunc, Roskild, Denmark) and treated with specific concentrations of HGF/SF (95% pure, Collaborative Research, Bedford, MA) for 24 hours. After two washes in PBS, cells were fixed for 10 min in cold methanol (−20°C) and washed extensively with PBS. Cells were also stained for 10 min with 0.1% methylene blue in PBS and were visualized and photographed with a Zeiss microscope.
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31. Fixed cells were embedded in L.R. gold resin (Electron Microscopy Sciences) at −25°C, sectioned with a Nova Ultratome (LKB, Uppsala, Sweden), and picked up with Formvar-coated 200-mesh gold grids (Fort Washington, PA). The grids were washed three times in PBS for 10 min and incubated in 1% bovine serum albumin (BSA) in PBS for 2 hours and in C28 antibody (diluted 1:50 in 1% BSA) at room temperature for 1 hour. Controls were incubated either in the presence of C28 competing peptide or in the absence of the primary antibody. The grids were washed again in PBS, incubated in RPMI 1640 medium for 20 min in 1% BSA, and reacted with goat antibody (to rabbit immunoglobulin) conjugated to gold (10-nm diameter; 1:10 diluted in 1% BSA; Amersham) at room temperature for 1 hour. The grids were finally washed in PBS and distilled water and stained with uranyl acetate and lead citrate. The sections were observed and photographed with an EM 410 electron microscope (Philips Eimdhoven, the Netherlands).

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Regulation of Protein Serine-Threonine Phosphatase Type-2A by Tyrosine Phosphorylation

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Extracellular signals that promote cell growth activate cascades of protein kinases. The kinases are dephosphorylated and deactivated by a single type-2A protein phosphatase. The catalytic subunit of type-2A protein phosphatase was phosphorylated by tyrosine-specific protein kinases. Phosphorylation was enhanced in the presence of the phosphatase inhibitor okadaic acid, consistent with an autodephosphorylation reaction. More than 90% of the activity of phosphatase 2A was lost when thioadenosine triphosphate was used to produce a thiophosphorylated protein resistant to autodephosphorylation. Phosphorylation in vitro occurred exclusively on Tyr^{307}. Phosphorylation was catalyzed by $p60^{v\text{-}src}$ $p56^{lck}$, epidermal growth factor receptors, and insulin receptors. Transient deactivation of phosphatase 2A might enhance transmission of cellular signals through kinase cascades within cells.

Protein phosphatase type-2A (phosphatase 2A) is one of the two predominant enzymes that hydrolyze phosphoserine and phosphothreonine residues in proteins in all eukaryotic cells (*1*). The catalytic subunit of phosphatase 2A is highly conserved among eukaryotic species. There is more than 70% identity in the amino acid sequences of phosphatase 2A from humans and the budding yeast *Saccharomyces cerevisiae*. The 309-residue protein is found in several species as two isoforms, which differ at eight positions (*2*). Phosphatase 2A is thought to exist in animal cells primarily as a heterotrimer (*3*), composed of three subunits, with A = 60 kD, B = 55 kD, and C (catalytic) = 36 kD. However, the AC heterodimer is specifically complexed with

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the middle T protein in polyomavirus-transformed cells (4). Other regulatory subunits of 70 kD and 50 kD have been reported as alternative components of heterotrimers (5). Although subunit-subunit interactions modulate the substrate specificity of phosphatase 2A, there are no accepted mechanisms for acute regulation of activity.

We purified the monomeric catalytic subunit of phosphatase 2A from rabbit skeletal muscle and the dimeric AC form from human erythrocytes (6). To test for in vitro phosphorylation, we isolated the Tyr kinase $p60^{v\text{-}src}$ by immunoprecipitation from IV5 cells (7) overexpressing the *v-src* gene (8) and incubated it with purified phosphatase 2A and γ-^{32}P-labeled adenosine triphosphate (ATP) (9). Recovery of the 36-kD phosphatase 2A catalytic subunit after the kinase reactions was the same at each time point on the basis of Coomassie staining of the gels. Autoradiography and densitometry of the 36-kD band showed that ^{32}P-labeling increased in a nearly linear fashion over time (Fig. 1A, open circles). Inclusion of okadaic acid (100 nM) to inhibit the activity of phosphatase 2A resulted in more rapid phosphorylation of the 36-kD catalytic subunit. Maximal phosphorylation occurred within 60 min (Fig. 1A). Calculations, based on Coomassie staining of protein and optical density of film exposure by ^{32}P with a standard curve, gave a stoichiometry of 0.82 to 0.92 phosphates per mole of phosphatase. Incorporation of ^{32}P after 60 min was 2.5-fold higher in the presence than in the absence of okadaic acid. Phosphorylation of the catalytic subunit was replicated in more than 12 independent experiments with five different phosphatase 2A preparations.

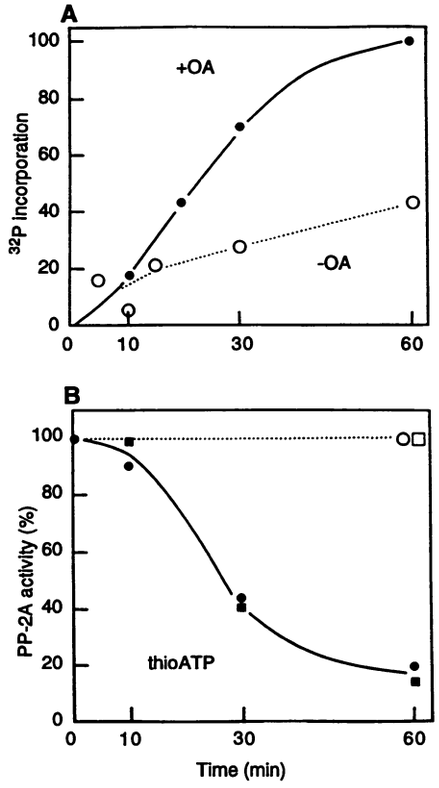

Fig. 1. Phosphorylation and inactivation of protein phosphatase type-2A by $p60^{v\text{-}src}$. (**A**) Incorporation of ^{32}P into 36-kD catalytic subunit of phosphatase 2A (PP-2A) in the presence (closed circles) and absence (open circles) of okadaic acid (OA) (100 nM). Tyrosine kinase $p60^{v\text{-}src}$ immunoprecipitated from IV5 cells was incubated with purified phosphatase 2A and [γ-^{32}P]ATP. During incubation, samples were withdrawn at timed intervals, were heated with SDS sample buffer, and were resolved by SDS-PAGE. Dried gels were exposed to Kodak X-AR film at room temperature for 7 hours. (**B**) Changes in phosphorylase phosphatase activity of phosphatase 2A after thiophosphorylation on the catalytic subunit of phosphatase 2A. Closed squares are the AC heterodimer form of phosphatase 2A, closed circles are the catalytic subunit. Shown in the graph are the averages of two independent experiments, each done in duplicate.

The activity of $p60^{v\text{-}src}$ was apparently unaffected by phosphatase 2A, because the ^{32}P-labeling of serum albumin and of immunoglobulin heavy chain in every reaction was the same, whether or not okadaic acid was added (*10*). Increased ^{32}P-labeling of phosphatase 2A in the presence of okadaic acid probably resulted from decreased autodephosphorylation rather than from changes in kinase activity. In other reactions we found that a longer incubation time or a higher amount of $p60^{v\text{-}src}$ yielded identical labeling of the 36-kD catalytic subunit, with or without okadaic acid. Therefore, okadaic acid affected the rate of accumulation of ^{32}P in the protein, not the amount of ^{32}P-labeled phosphatase 2A produced. It is possible that binding of okadaic acid changed the conformation of the phosphatase into a better substrate for $p60^{v\text{-}src}$. However, we favor the conclusion that autodephosphorylation occurs and that the increased labeling in the presence of okadaic acid is due to inhibition of phosphatase activity during the kinase reaction. We have observed loss of ^{32}P from the isolated catalytic subunit after transfer onto nitrocellulose, consistent with an intramolecular dephosphorylation. The results also predict that phosphatase activity was diminished as a result of the phosphorylation.

Changes in phosphatase 2A activity caused by phosphorylation by $p60^{v\text{-}src}$ could not be determined as long as the potent inhibitor okadaic acid was present in the reactions. However, when γ-thioATP is substituted for ATP, kinases form thiophosphorylated residues in proteins, which are relatively resistant to protein phosphatases (*11*). Reaction mixtures containing γ-thioATP were sampled at various times. We determined phosphatase activity by measuring the release of ^{32}P from Ser^{14} in the exogenous substrate phosphorylase a (*12*). Under these conditions the phosphatase 2A catalytic subunit or AC heterodimer was progressively inactivated. More than 90% of phosphorylase phosphatase activity was lost within 1 hour (Fig. 1B). Phosphatase 2A activity was unchanged and fully sensitive to okadaic acid (100 nM) in reactions that were otherwise identical, except for omission of γ-thioATP or omission of the

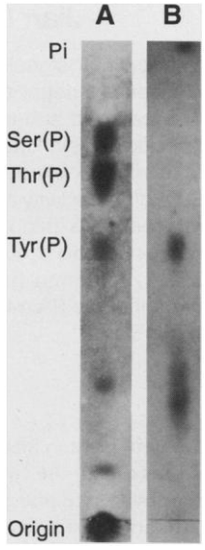

Fig. 2. Phosphoamino acid analysis of phosphatase 2A catalytic subunit phosphorylated in vitro by $p60^{v\text{-}src}$. Phosphorylated phosphatase 2A was acid-hydrolyzed, phosphoamino acid standards [phosphoserine, Ser(P); phosphothreonine, Thr(P); and phosphotyrosine, Tyr(P)] were added, and separation was accomplished by electrophoresis at pH 3.5 as described (*14*). The plate was stained with ninhydrin and was photographed (**A**). The plate was exposed to Kodak X-AR film at −70°C for 3 days with Cronex Lightning-plus (DuPont) enhancing screens, and a photograph of the film is shown in (**B**). Some ^{32}P inorganic phosphate (Pi) was detected near the top of the plate, and some incompletely hydrolyzed ^{32}P-peptides were detected near the bottom, but Tyr(P) was the only phosphoamino acid detected.

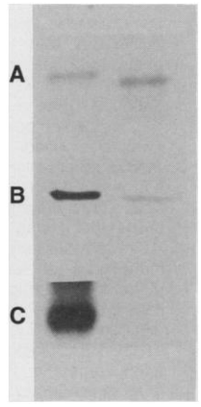

Fig. 3. Removal of the tyrosine phosphorylation site by digestion of the protein phosphatase type-2A COOH-terminus with trypsin. Samples of phosphatase 2A undigested (left lanes) or digested 15 min at 30°C with trypsin (0.1 μg) at pH 8 (right lanes) were compared. (**A**) Coomassie-stained polyacrylamide gel. (**B**) Protein immunoblot with affinity-purified antipeptide antibody to the COOH-terminal 12 amino acids of phosphatase 2A. (**C**) Autoradiogram of catalytic subunit after phosphorylation by $p60^{v\text{-}src}$ in the presence of [γ-^{32}P]ATP (*8*). The Coomassie staining and the autoradiogram represent the same lanes of a single gel. The same results were obtained in three independent experiments with different phosphatase 2A preparations.

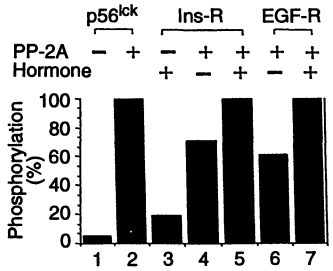

Fig. 4. Phosphorylation of protein phosphatase type-2A catalytic subunit by protein tyrosine kinases. Reactions were carried out as described (*8*), except that different kinases were substituted for the $p60^{v\text{-}src}$. Samples 1 and 3 contained no added phosphatase 2A. Labeling of phosphatase 2A (PP-2A) by receptors for insulin (Ins-R) and EGF (EGF-R) (lanes 4 and 6, respectively) was enhanced by addition of cognate hormones (1 μg/ml) (lanes 5 and 7, respectively). Results similar to this representative experiment were observed in three other experiments.

$p60^{v\text{-}src}$ kinase (Fig. 1B, open square and open circle).

Although $p60^{v\text{-}src}$ is a Tyr-specific kinase (*13*), it was important to demonstrate that Tyr was phosphorylated in phosphatase 2A and to establish the site of phosphorylation within the protein. The catalytic subunit of phosphatase 2A was ^{32}P-labeled, was excised from a polyacrylamide gel, and was digested with trypsin. Only one ^{32}P-labeled tryptic peptide was detected after two-dimensional thin-layer electrophoresis and chromatography (*10*). Acid hydrolysis and high-voltage electrophoresis on cellulose thin-layer plates (*14*) showed that all of the phosphorylation of phosphatase 2A occurred on Tyr residues (Fig. 2). The high yield of phosphotyrosine compared to inorganic phosphate and the instability in acid of phosphotyrosine relative to phosphoserine or phosphothreonine (*15*) reinforce this conclusion.

The site of $p60^{v\text{-}src}$ phosphorylation lies within a peptide which can be removed by trypsin from the COOH-terminus of phosphatase 2A, thus identifying it as Tyr^{307}. No Tyr residues are near the NH_2-terminus, the first being at position 80 in the sequence (*16*). However, at two residues from the COOH-terminus, there is a Tyr residue within the sequence Arg-Arg-Thr-Pro-Asp-Tyr-Phe-Leu that is conserved among all eukaryotic phosphatase 2As, but it is completely different from the COOH-terminus of type-1 phosphatases.

Digestion of heteromeric phosphatase 2A or free C subunit monomer with trypsin resulted in cleavage at one dominant site in the C subunit, with a reduction in size from 36 to 35 kD that was discernible by polyacrylamide gel electrophoresis (PAGE) (Fig. 3). Only the full-length, 36-kD C subunit, but not the 35-kD, trypsin-truncated form of phosphatase 2A, was phosphorylated by $p60^{v\text{-}src}$ (Fig. 3C). Consistent with the loss of phosphorylation, the trypsin-truncated form was not inactivated like the full-length phosphatase 2A by reaction with ATP plus $p60^{src}$. Cleavage was at the tandem arginines at residues 302 and 303 or 10 residues earlier at arginines residues 293 and 294 (*17*); in either case only one Tyr residue is removed. The same amount of full-length and truncated protein was visualized with Coomassie staining (Fig. 3A). Antibodies were raised to a peptide corresponding to 12 COOH-terminal residues of phosphatase 2A. This antibody bound less efficiently to the trypsin-truncated, 35-kD subunit than to the 36-kD, full-length protein (Fig. 3B). In another study partial trypsin digestion of phosphatase 2A did not reduce binding of an antibody to the COOH-terminal 20 residues but did eliminate binding of antibody to the COOH-terminal 10 residues (*17*).

Phosphatase 2A also was phosphorylated by other receptor and nonreceptor protein Tyr kinases. The src-related kinase $p56^{lck}$ from transformed T cells, insulin receptors purified from human placenta, and epidermal growth factor (EGF) receptors purified from human epidemoid carcinoma A431 cells (*18*) all catalyzed phosphorylation of the 36-kD phosphatase 2A catalytic subunit (Fig. 4). Phosphorylation was quantitated by densitometry of autoradiograms after SDS-PAGE. Appearance of a ^{32}P-labeled band at 36 kD was dependent on addition of phosphatase 2A (Fig. 4) and was enhanced by the addition of either insulin or EGF, showing that the phosphorylation was dependent on hormone activation of the receptors.

Several protein kinases that participate in mitogenic and hormone signaling, such as ribosomal protein S6 kinases (RSKs) (*19*) and mitogen-activated protein (MAP) kinases ($p42^{MAP}$, $p54^{MAP}$, and $p44^{mpk}$) (*20*), kinases that activate MAP kinases (*21*), and other insulin-stimulated kinases (*22*), all are inactivated by phosphatase 2A. The Tyr phosphorylation of phosphatase 2A might enhance the cascade of phosphorylation in response to cell stimulation. Because removal of the COOH-terminal segment did not affect phosphatase 2A activity, but phosphorylation of Tyr^{307} diminished phosphatase 2A activity, it is possible that phosphorylated Tyr^{307} occupies the active site. Autodephosphorylation of phosphatase 2A provides a mechanism for quantitative reactivation of the phosphatase without requiring additional proteins. If temporary inactivation of phosphatase 2A is involved in mitogen activation of cells, this might explain the tumor-promoting effects of phosphatase inhibitors such as okadaic acid (*23*). The 36-kD catalytic subunit was phosphorylated in vivo on Tyr in IV5 cells overexpressing $p60^{v\text{-}src}$, in peripheral blood lymphocytes activated by antibody cross-linking of surface markers, and in middle T immunocomplexes from transformed cells (*10*). Transformation of cells by proteins such as middle T and $p60^{v\text{-}src}$ may require in vivo Tyr phosphorylation of phosphatase 2A.

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9. Kinase reactions included the 36-kD catalytic subunit of phosphatase 2A (1 μg) or the phosphatase 2A heterodimer (3 μg) (36-kD catalytic subunit and 60-kD regulatory subunit), kinase-buffer [25 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 0.1% NP-40, 60 mM 2-mercaptoethanol, 100 mM NaCl, 10% glycerol], bovine serum albumin (1 μg), 0.2 mM ATP, 22.5 μCi of γ-^{32}P-labeled ATP, and Protein A–Sepharose beads (4 μl) (Sigma, St. Louis, MO) bound to $p60^{v\text{-}src}$ in a total reaction volume of 10 μl.
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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

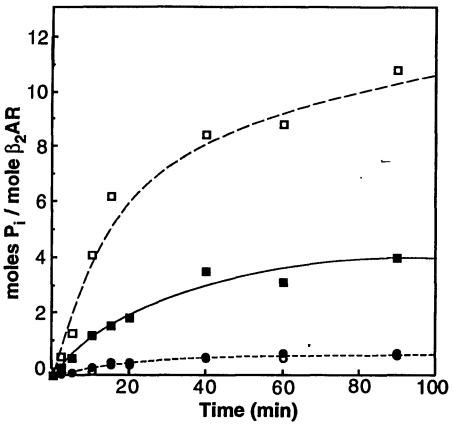

Fig. 1. Enhancement of βARK-mediated β_2AR phosphorylation by bovine brain βγ. Human β_2AR and bovine βARK were expressed in Sf9 cells and were purified with modifications of published procedures (*11*, *12*). The partially purified receptor was reconstituted into phospholipid vesicles (*15*, *16*) and 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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