β-Arrestin–Dependent Formation of β_2 Adrenergic Receptor–Src Protein Kinase Complexes

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The Ras-dependent activation of mitogen-activated protein (MAP) kinase pathways by many receptors coupled to heterotrimeric guanine nucleotide binding proteins (G proteins) requires the activation of Src family tyrosine kinases. Stimulation of β_2 adrenergic receptors resulted in the assembly of a protein complex containing activated c-Src and the receptor. Src recruitment was mediated by β-arrestin, which functions as an adapter protein, binding both c-Src and the agonist-occupied receptor. β-Arrestin 1 mutants, impaired either in c-Src binding or in the ability to target receptors to clathrin-coated pits, acted as dominant negative inhibitors of β_2 adrenergic receptor–mediated activation of the MAP kinases Erk1 and Erk2. These data suggest that β-arrestin binding, which terminates receptor–G protein coupling, also initiates a second wave of signal transduction in which the "desensitized" receptor functions as a critical structural component of a mitogenic signaling complex.

The basic unit of G protein–coupled receptor (GPCR) signaling consists of three parts: a heptahelical receptor that detects ligands in the extracellular milieu, a G protein that dissociates into α subunits bound to guanosine triphosphate (GTP) and βγ subunits after interaction with the ligand-bound receptor, and an effector that interacts with dissociated G protein subunits to generate small-molecule second messengers. The receptor–G protein interaction is catalytic; that is, one receptor sequentially activates multiple G proteins.

The termination of GPCR signals involves binding of proteins to the receptor. This process is initiated by serine-threonine phosphorylation of agonist-occupied receptors, both by members of the G protein–coupled receptor kinase (GRK) family and by second-messenger–activated protein kinases such as adenosine 3′,5′-monophosphate–dependent protein kinase (PKA) and protein kinase C. Receptor phosphorylation by GRKs is followed by binding of proteins termed arrestins, which bind the phosphorylated receptor and sterically inhibit further G protein activation (*1*). Desensitized receptor-arrestin complexes undergo arrestin-dependent targeting for sequestration through clathrin-coated pits (*2, 3*). Sequestered receptors are ultimately either dephosphorylated and recycled to the cell surface or targeted for degradation (*4*).

Many GPCRs mediate Ras-dependent activation of mitogenic signaling pathways through mechanisms similar to those that mediate signaling by receptor tyrosine kinases (RTKs). Although the activation of G proteins is clearly necessary, several lines of evidence indicate that the classical model of GPCR signaling is insufficient to account for these Ras-dependent signals. In fibroblasts, GPCR-mediated stimulation of the MAP kinases Erk1 and Erk2 is dissociable from the activation of G protein effectors such as phospholipase C and adenylyl cyclase (*5*). Rather, GPCR-mediated activation of Erks requires tyrosine protein phosphorylation and assembly of a membrane-associated Ras activation complex. Stimulation of receptors coupled to members of the G_i and G_q classes of G protein α subunit induces rapid tyrosine phosphorylation of the Shc and Gab1 adapter proteins, followed by Grb2-dependent recruitment of the Ras guanine nucleotide exchange factor mSos1 (*6, 7*).

Recruitment and activation of Src family nonreceptor tyrosine kinases is required for GPCR-mediated activation of Ras. Stimulation of Src, Fyn, Yes, or Lyn activity by several GPCRs has been reported (*8, 9*), and inhibition of Src kinase activity impairs lysophosphatidic acid (LPA) and β_2 adrenergic receptor (β_2AR)–mediated tyrosine phosphorylation of Shc and Gab1, formation of Shc-Grb2 complexes, and activation of Erks (*7, 9, 10*).

Activation of Erks by β_2ARs is also dependent on receptor desensitization and sequestration, processes generally regarded as signal termination events. In HEK-293 cells, PKA-mediated phosphorylation of the β_2AR confers receptor coupling to G_i, with subsequent stimulation of Erks mediated through activation of G_i (*10*). Further, cellular expression of dominant inhibitory mutants of β- arrestin 1 or dynamin, which inhibit agonist-induced receptor sequestration, block β_2AR-mediated activation of Erks, with no effect on receptor-mediated second messenger generation (*11*).

Agonist-promoted formation of a protein complex containing the β_2 adrenergic receptor, β-arrestin 1, and c-Src. In unstimulated fibroblasts, about 90% of the Src is associated with intracellular vesicle membranes, away from the plasma membrane. Upon activation of RTKs, 5 to 10% of the Src redistributes either to the plasma membrane or to the cytoskeleton (*12*). To determine whether β_2AR activation induces the redistribution of Src, we examined the effects of isoproterenol stimulation on the cellular distribution of endogenous Src in cells transiently expressing hemagglutinin (HA) epitope–tagged β_2ARs.

The distribution of β_2AR, β-arrestin, and Src in HEK-293 cells was determined by confocal immunofluorescence microscopy before and after exposure to agonist (Fig. 1). Before stimulation, the β_2ARs were organized into plasma membrane clusters by cross-linking with a polyclonal rabbit antibody to HA; this was done to facilitate visualization of β_2ARs in the absence of agonist and to prevent their agonist-induced translocation to clathrin-coated pits. In the absence of agonist, β-arrestin staining was predominantly cytosolic, with no distinct aggregates corresponding to the distribution of the β_2AR clusters (Fig. 1A). After exposure of cells to isoproterenol for 5 min, a portion of immunoreactive β-arrestin appeared in plasma membrane clusters that coincided with the distribution of β_2ARs, indicative of agonist-dependent translocation of β-arrestin to the receptor.

Activated Src was visualized with a monoclonal antibody (mAb), Clone 28, which specifically recognizes the activated form of c-Src dephosphorylated at COOH-terminal residue Tyr^{530} (*13*). In the absence of agonist, activated Src was found primarily

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associated with cytoskeletal components, and no substantial overlap of receptor and Src existed (Fig. 1B). After a 5-min exposure to agonist, a portion of the activated Src appeared in plasma membrane clusters that coincided precisely with the distribution of the β_2ARs. Thus, like β-arrestins, endogenous Src undergoes an agonist-dependent redistribution to localize with β_2ARs.

Endogenous β-arrestin and c-Src coprecipitated with Flag epitope–tagged β_2ARs from transfected HEK-293 cells after exposure of the cells to agonist and covalent cross-linking of receptor-associated proteins (Fig. 2A). β-Arrestin 1 and c-Src exhibited a similar time course of receptor association, with complex formation increasing by about a factor of 5 within 2 to 5 min of exposure to isoproterenol.

Agonist-dependent recruitment of Src to β_2ARs might represent direct association of the kinase and receptor. Alternatively, it might reflect binding of the kinase to a third protein, such as β-arrestin, that binds the receptor in an agonist-dependent manner. Because COS-7 cells express small amounts of endogenous β-arrestins (*14*), we used them to determine whether overexpression of β-arrestin would enhance association of c-Src with Flag epitope–tagged β_2ARs. Little agonist-induced binding of endogenous β-arrestins or c-Src to the receptor was detectable in COS-7 cells in the absence of overexpressed β-arrestin 1 (Fig. 2B). Increases in receptor-bound β-arrestin (by a factor of 2 to 3) were detectable after 1 min of agonist exposure in cells overexpressing wild-type β-arrestin 1. In the absence of excess β-arrestin 1, overexpression of wild-type c-Src was not sufficient to produce detectable association of c-Src with the β_2AR. However, when both wild-type c-Src and β-arrestin 1 were overexpressed, agonist treatment resulted in an increase (by a factor of 3 to 4) in the amount of c-Src present in the β_2AR immunoprecipitate. The stoichiometric ratio of c-Src to β-arrestin 1 in these receptor immunoprecipitates was 0.84 (±0.15):1 (n = 6), consistent with the exclusive association of c-Src with β-arrestin–bound receptors. In contrast, angiotensin II type 1A (AT1A) receptors, which internalize in a β-arrestin–independent manner (*15*), did not form agonist-induced complexes with c-Src under identical conditions (*16*). The intrinsic tyrosine kinase activity of c-Src was not required for the formation of c-Src–β-arrestin 1–β_2AR complexes because a catalytically inactive mutant c-Src (Lys^{298} → Met, or K298M) was recruited into the complex as effectively as wild-type c-Src.

β-Arrestin 1 and c-Src immunoprecipitates from whole-cell detergent lysates of transfected COS-7 cells each contained the other protein, suggesting that the β-arrestin–dependent association of c-Src with the receptor reflected the formation of β-arrestin 1–c-Src complexes (Fig. 2C). Isoproterenol stimulation increased the association of β-arrestin 1 and c-Src by about a factor of 2.

Activation of c-Src bound to β-arrestin 1. Intramolecular binding of the Src homology 2 (SH2) domain of c-Src to a phosphorylated tyrosine residue within its COOH-terminus (Tyr^{530}) suppresses c-Src kinase activity (*17*). Activation of c-Src is often associated with dephosphorylation of this COOH-terminal regulatory tyrosine. Protein binding to the c-Src SH2 or SH3 domains may also produce conformational changes that increase kinase activity. For example, binding of the c-Src SH3 domain to a peptide fragment of the Crk-associated substrate $p130^{Cas}$-related protein, Sin, is sufficient to induce activation of c-Src (*18*). Similarly, binding of the human immunodeficiency virus–type 1 Nef protein to the SH3

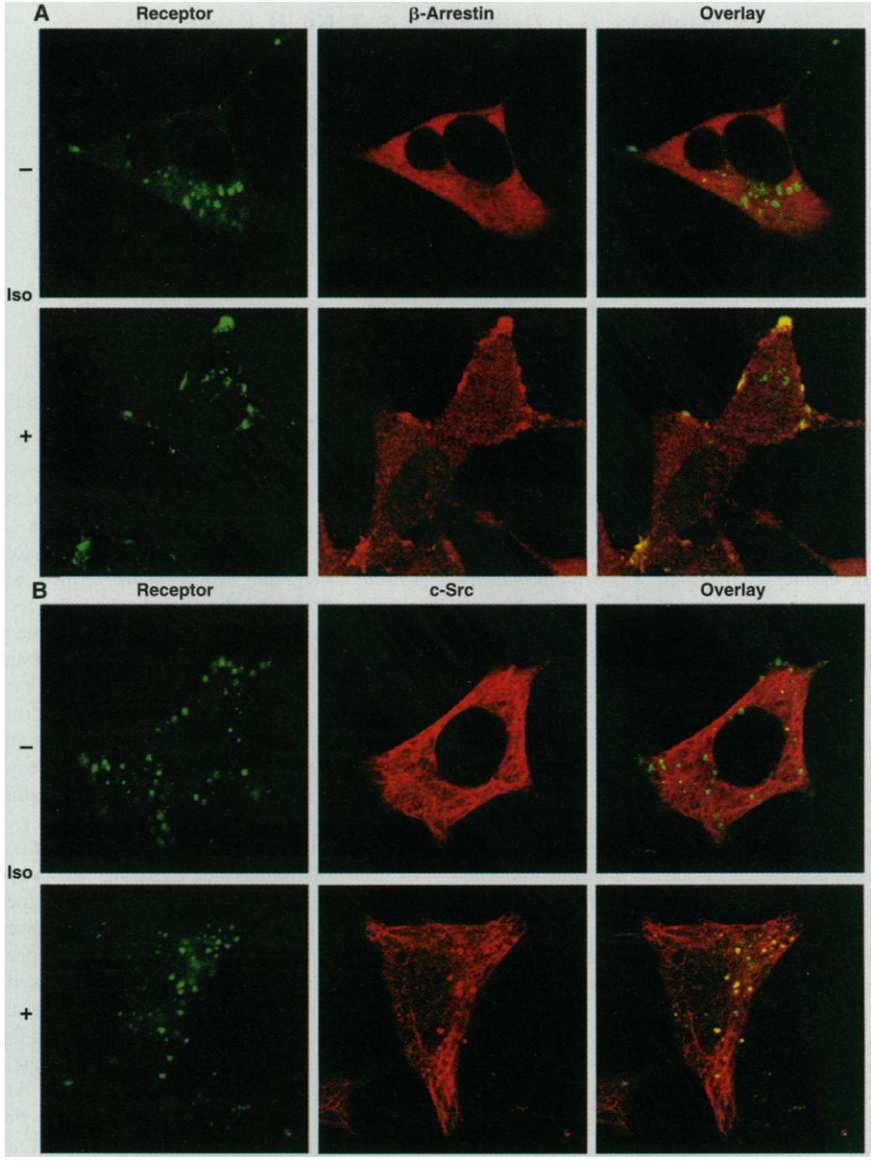

Fig. 1. Localization of β-arrestin and activated Src with β_2ARs after agonist exposure. HEK-293 cells transiently expressing HA epitope–tagged β_2ARs were grown on ethanol-washed cover slips (*35*). Epitope-tagged receptors were organized into plasma membrane clusters by cross-linking with a primary layer of rabbit polyclonal antibody to the HA epitope, followed by a secondary layer of fluorescein-conjugated goat antibody to rabbit IgG. Cells were stimulated for 5 min at 37°C in the absence or presence of isoproterenol (10 μM), then fixed, permeabilized, and labeled with either a primary β-arrestin 1 mAb (**A**) or a primary mAb (Clone 28) raised against Tyr^{530}-dephosphorylated c-Src (**B**), followed by a secondary layer of Texas Red–conjugated goat antibody to mouse IgG (*27, 36*). Shown are representative confocal microscopic images depicting the cellular distribution of β_2ARs (left panels) and β-arrestin 1 or Tyr^{530}-dephosphorylated c-Src (center panels). Overlay images (right panels) depict colocalization of β_2ARs and β-arrestin or c-Src in the presence of agonist (yellow).

domain of the Src family tyrosine kinase Hck increases the specific activity of the kinase in vitro (*19*).

The clone 28 antibody to dephosphorylated Src recognizes c-Src present in β-arrestin 1 immunoprecipitates (Fig. 3A). The specific activity of c-Src in β-arrestin 1 immunoprecipitates was 8.8 times the activity measured in whole-cell c-Src immunoprecipitates from HEK-293 cells transiently overexpressing both c-Src and β-arrestin 1 (*20*), suggesting that the c-Src associated with β-arrestin 1 is an activated form of the kinase (Fig. 3B).

Because the c-Src bound to β-arrestin 1 is substantially dephosphorylated at Tyr^{530}, these data do not distinguish whether binding results in conformational activation of the kinase, or whether β-arrestin 1 simply binds preferentially to dephosphorylated c-Src. To distinguish these alternatives, we determined whether β-arrestin 1 binding affected the specific activity of the Tyr^{530} → Phe (Y530F) mutant of c-Src, in which the regulatory COOH-terminal phosphorylation site has been mutated to phenylalanine, or of the retroviral oncogene product v-Src, which lacks the COOH-terminal regulatory domain. Binding to β-arrestin 1 increased the relative specific activity of both Y530F c-Src and v-Src (Fig. 3B), consistent with kinase activation resulting from a conformational change induced by β-arrestin 1 binding independent of the phosphorylation state of the c-Src COOH-terminus.

Binding of c-Src to the β-arrestin 1 NH_2-terminus. To determine the region of β-arrestin 1 that interacts with c-Src, we used a series of Flag epitope–tagged β-arrestin 1 deletion or truncation mutants (Fig. 4A). Wild-type or mutant β-arrestin 1 was expressed in COS-7 cells along with wild-type c-Src, and coprecipitation of β-arrestin 1–bound c-Src was monitored by immunoblotting. Deletion of amino acids 1 to 185 from the NH_2-terminus of β-arrestin 1 resulted in the complete loss of c-Src binding (Fig. 4B). Conversely, a β-arrestin 1 fragment that comprised amino acids 1 to 163 interacted with c-Src as efficiently as did wild-type β-arrestin 1, indicating that c-Src binds to the β-arrestin 1 NH_2-terminus.

To determine the region of c-Src that binds β-arrestin 1, we assessed the ability of glutathione S-transferase (GST) fusion proteins containing either the c-Src SH2 or SH3 domain (Fig. 4C) to bind purified recombinant histidine epitope (His^6)–tagged β-arrestin 1 in vitro. Immunoprecipitates of His^6-tagged β-arrestin 1 coprecipitated the GST–Src SH3 domain or purified recombinant c-Src, but not the GST–Src SH2 domain (Fig. 4D). The binding of recombinant c-Src to His^6-tagged β-arrestin 1 was inhibited in the presence of excess GST–Src SH3 domain but not GST–Src SH2 domain, suggesting that the c-Src SH3 domain contributes to the binding of the two intact proteins.

SH3 domains mediate hydrophobic interactions with proteins, most of which contain the minimal consensus sequence Pro-X-X-Pro (where X represents any amino acid) (*21*). β-Arrestin 1 contains three clusters of proline residues within its NH_2-terminus that conform to this motif (Fig. 5A). A point mutation of β-arrestin 1 created by site-directed mutagenesis of two proline residues within the NH_2-terminus, Pro^{91} → Gly and Pro^{121} → Glu (P91G-P121E β-arrestin 1), reduced c-Src binding (Fig. 5B). In contrast, another NH_2-terminal point mutation of β- arrestin 1, Val^{53} → Asp (V53D β-arrestin 1), which inhibits

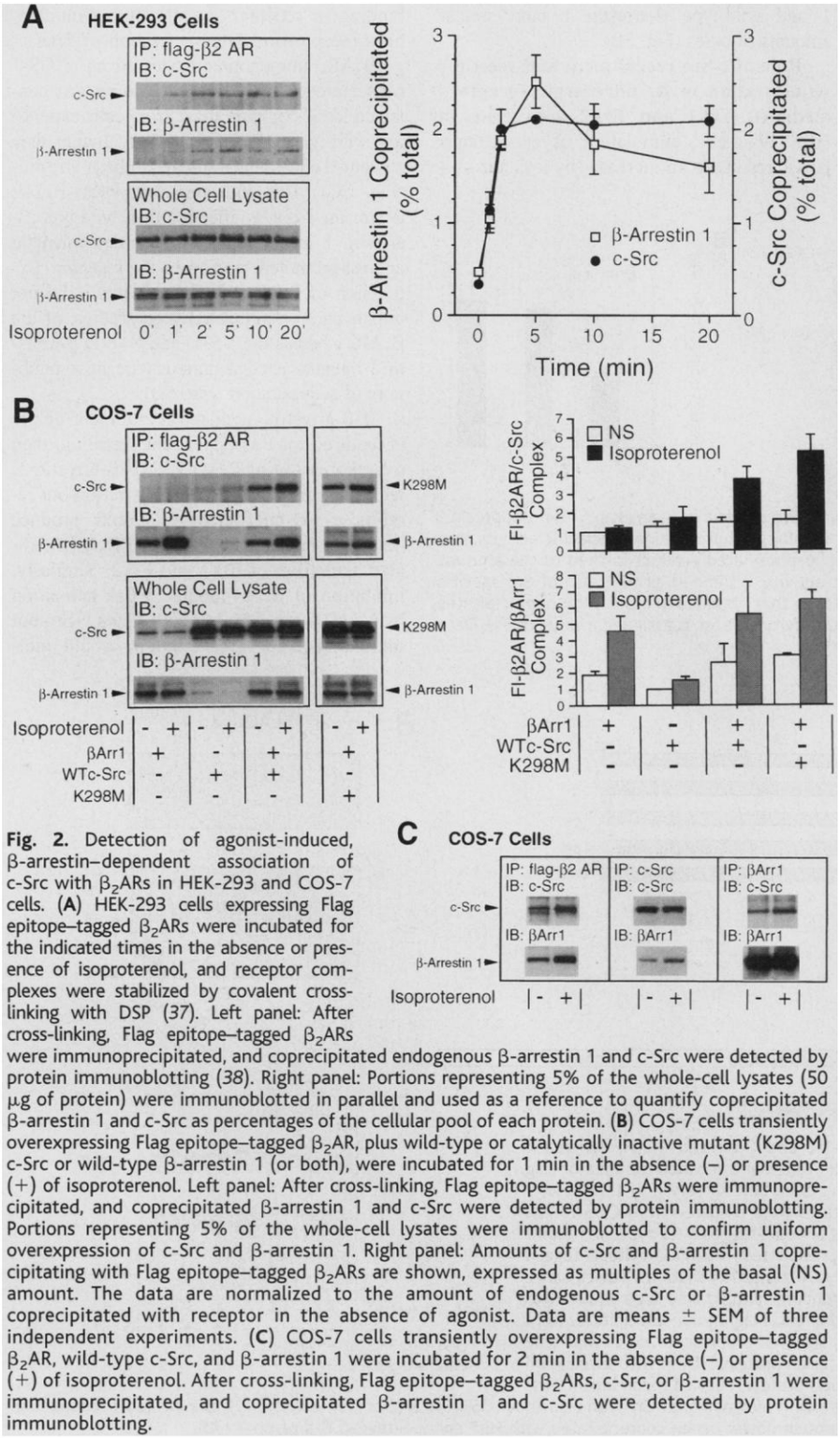

Fig. 2. Detection of agonist-induced, β-arrestin–dependent association of c-Src with β_2ARs in HEK-293 and COS-7 cells. (**A**) HEK-293 cells expressing Flag epitope–tagged β_2ARs were incubated for the indicated times in the absence or presence of isoproterenol, and receptor complexes were stabilized by covalent cross-linking with DSP (*37*). Left panel: After cross-linking, Flag epitope–tagged β_2ARs were immunoprecipitated, and coprecipitated endogenous β-arrestin 1 and c-Src were detected by protein immunoblotting (*38*). Right panel: Portions representing 5% of the whole-cell lysates (50 μg of protein) were immunoblotted in parallel and used as a reference to quantify coprecipitated β-arrestin 1 and c-Src as percentages of the cellular pool of each protein. (**B**) COS-7 cells transiently overexpressing Flag epitope–tagged β_2AR, plus wild-type or catalytically inactive mutant (K298M) c-Src or wild-type β-arrestin 1 (or both), were incubated for 1 min in the absence (–) or presence (+) of isoproterenol. Left panel: After cross-linking, Flag epitope–tagged β_2ARs were immunoprecipitated, and coprecipitated β-arrestin 1 and c-Src were detected by protein immunoblotting. Portions representing 5% of the whole-cell lysates were immunoblotted to confirm uniform overexpression of c-Src and β-arrestin 1. Right panel: Amounts of c-Src and β-arrestin 1 coprecipitating with Flag epitope–tagged β_2ARs are shown, expressed as multiples of the basal (NS) amount. The data are normalized to the amount of endogenous c-Src or β-arrestin 1 coprecipitated with receptor in the absence of agonist. Data are means ± SEM of three independent experiments. (**C**) COS-7 cells transiently overexpressing Flag epitope–tagged β_2AR, wild-type c-Src, and β-arrestin 1 were incubated for 2 min in the absence (–) or presence (+) of isoproterenol. After cross-linking, Flag epitope–tagged β_2ARs, c-Src, or β-arrestin 1 were immunoprecipitated, and coprecipitated β-arrestin 1 and c-Src were detected by protein immunoblotting.

β_2AR sequestration (*2*), had no effect on the interaction of β-arrestin 1 and c-Src.

These data indicate that c-Src interacts with hydrophobic domains within the NH_2-terminus of β-arrestin 1. However, the formation of β-arrestin 1–c-Src complexes is also modulated by agonist-induced dephosphorylation of the COOH-terminus of β- arrestin 1. Cytosolic β-arrestin 1 is predominantly phosphorylated on a COOH-terminal serine residue, Ser^{412} (*22*). β_2AR activation promotes the translocation of Ser^{412}-phosphorylated β-arrestin 1 from the cytosol to the receptor, where it is dephosphorylated. Mutants of β-arrestin 1 that mimic the phosphorylated or dephosphorylated forms, Ser^{412} → Asp (S412D) and Ser^{412} → Ala (S412A) respectively, bind equivalently to agonist- occupied receptor. S412D β-arrestin 1, however, does not bind clathrin and thus acts as a dominant negative inhibitor of receptor sequestration. S412D β-arrestin 1 also causes a loss of c-Src binding despite the presence of an intact NH_2-terminus, whereas S412A β-arrestin 1 and wild-type β-arrestin 1 bind similar amounts of c-Src (Fig. 5B).

Role of c-Src recruitment and receptor sequestration in β_2 adrenergic receptor–mediated Erk1 and Erk2 activation. In HEK-293 cells, stimulation of endogenous β_2AR results in an increase (by a factor of 4 to 5) in the phosphorylation of Erk1 and Erk2, which is maximal within 5 min of stimulation. To determine whether the assembly of Src–β-arrestin–receptor complexes or β-arrestin–dependent receptor sequestration is required for Ras-dependent signaling through β_2ARs, we tested whether β-arrestin 1 mutants selectively defective in either c-Src binding or receptor sequestration would inhibit receptor-mediated activation of Erks.

β_2AR immunoprecipitates from COS-7 cells expressing P91G-P121E β-arrestin 1 contained less c-Src than those from cells expressing wild-type β-arrestin 1, even though they contained equivalent amounts of the β-arrestins (Fig. 5C). This indicates that P91G-P121E β-arrestin 1 is less effective than wild-type β-arrestin 1 in binding c-Src, but equivalent in agonist-promoted binding to the receptor. Expression of P91G-P121E β-arrestin 1 did not impair agonist-stimulated sequestration of the β_2AR, whereas the V53D and S412D β-arrestin 1 mutants acted as dominant negative inhibitors of sequestration (Fig. 5D) (*2, 22*).

If β-arrestin–mediated recruitment of Src is required for Ras-dependent signaling, then overexpression of P91G-P121E β-arrestin 1, which supports receptor sequestration but recruits c-Src inefficiently, should produce dominant negative inhibition of β_2AR-mediated activation of Erk1 and Erk2. Similarly, inhibition of β_2AR-mediated Erk activation by V53D β-arrestin 1, which binds c-Src but inhibits receptor sequestration, would indi-

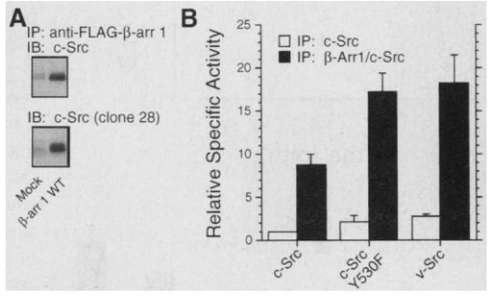

Fig. 3. Effect of β-arrestin 1 binding on the specific activity of c-Src. (**A**) Clone 28 immunoblot of Tyr^{530}-dephosphorylated c-Src coprecipitated with wild-type Flag epitope–tagged β-arrestin 1. (**B**) Specific activity of β-arrestin 1–bound Src. HEK-293 cells were transiently transfected with Flag epitope–tagged β-arrestin 1 plus wild-type c-Src, Y530F c-Src, or v-Src. In vitro Src kinase assays (*20*) were done on parallel β-arrestin 1 and c-Src immunoprecipitates. The amount of Src present in each kinase reaction was determined by protein immunoblotting. The quantity of Src present in β-arrestin 1 immunoprecipitates typically represented about 10% of the total cellular pool of the kinase. Specific activity was calculated as the ratio of the amount of tyrosine-phosphorylated product formed to the amount of kinase present in the reaction. Relative specific activity is defined as the ratio of Src specific activity measured in β-arrestin 1 immunoprecipitates to that obtained in c-Src immunoprecipitates from identically transfected cells, where the specific activity of c-Src is assigned a value of 1.0. Data are means ± SEM of three independent experiments.

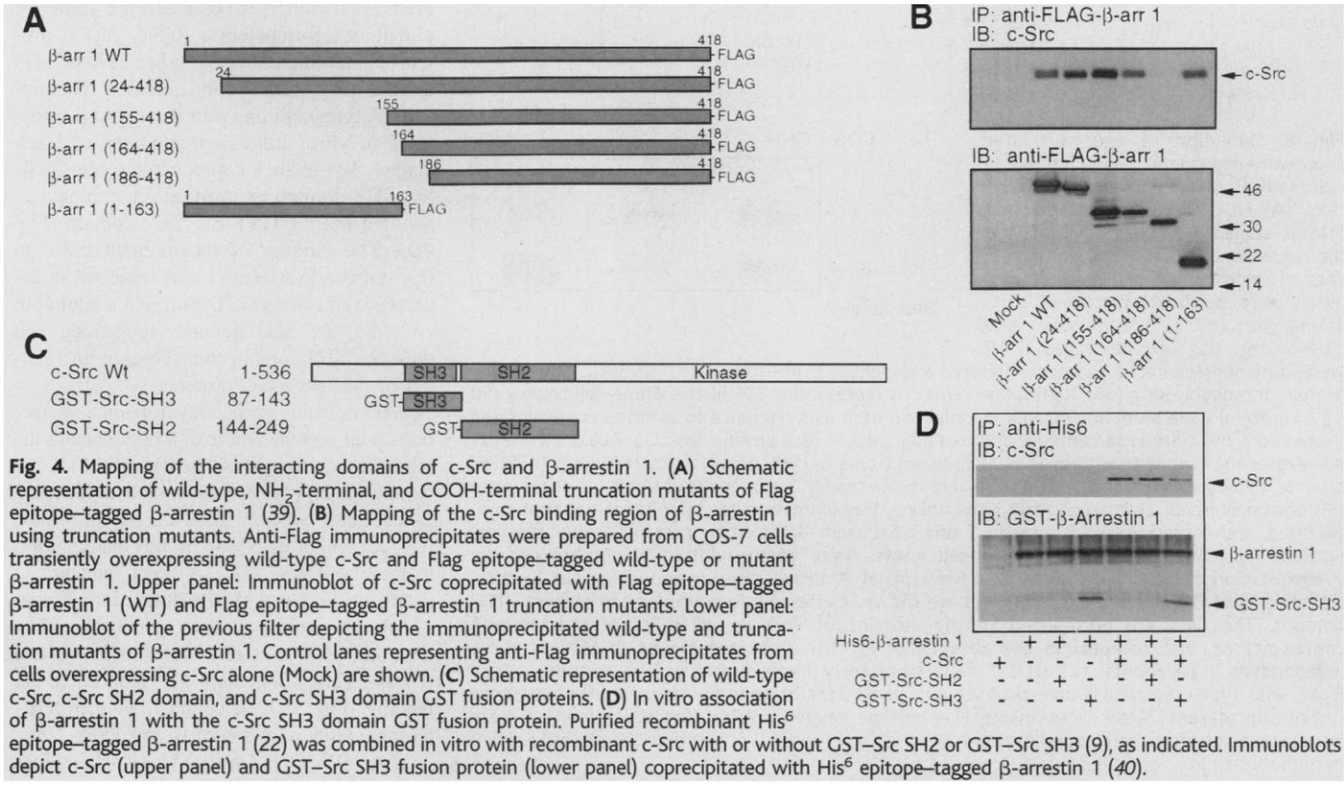

Fig. 4. Mapping of the interacting domains of c-Src and β-arrestin 1. (**A**) Schematic representation of wild-type, NH_2-terminal, and COOH-terminal truncation mutants of Flag epitope–tagged β-arrestin 1 (*39*). (**B**) Mapping of the c-Src binding region of β-arrestin 1 using truncation mutants. Anti-Flag immunoprecipitates were prepared from COS-7 cells transiently overexpressing wild-type c-Src and Flag epitope–tagged wild-type or mutant β-arrestin 1. Upper panel: Immunoblot of c-Src coprecipitated with Flag epitope–tagged β-arrestin 1 (WT) and Flag epitope–tagged β-arrestin 1 truncation mutants. Lower panel: Immunoblot of the previous filter depicting the immunoprecipitated wild-type and truncation mutants of β-arrestin 1. Control lanes representing anti-Flag immunoprecipitates from cells overexpressing c-Src alone (Mock) are shown. (**C**) Schematic representation of wild-type c-Src, c-Src SH2 domain, and c-Src SH3 domain GST fusion proteins. (**D**) In vitro association of β-arrestin 1 with the c-Src SH3 domain GST fusion protein. Purified recombinant His^6 epitope–tagged β-arrestin 1 (*22*) was combined in vitro with recombinant c-Src with or without GST–Src SH2 or GST–Src SH3 (*9*), as indicated. Immunoblots depict c-Src (upper panel) and GST–Src SH3 fusion protein (lower panel) coprecipitated with His^6 epitope–tagged β-arrestin 1 (*40*).

cate a requirement for targeting of the receptor–β-arrestin 1–c-Src complex to clathrin-coated pits. Phosphorylation of Erks in response to activation of endogenous β_2ARs was inhibited by 60 to 70% in HEK-293 cells transiently expressing either P91G-P121E β-arrestin 1 or V53D β-arrestin 1 (Fig. 6A). In contrast, epidermal growth factor (EGF)–mediated phosphorylation of Erks was not affected. Similar results were obtained when comparing the effects of S412A β-arrestin 1 and S412D β-arrestin 1. S412D β-arrestin 1, which is impaired in its ability to bind c-Src and to support receptor sequestration, inhibited β_2AR-mediated phosphorylation of Erks.

To determine whether β-arrestin function is required for activation of Erks through other GPCRs, we determined the effects of the β-arrestin 1 mutants on activation of Erks by transiently expressed serotonin 5HT1A and AT1A receptors. Like β_2ARs, 5HT1A receptors mediate pertussis toxin–sensitive activation of Erks by a mechanism dependent on G protein βγ subunits (*23*). AT1A receptors also activate Erks through a Ras-dependent pathway that requires Src (*24*); however, these receptors signal through pertussis toxin–insensitive G proteins and internalize in a β-arrestin–independent manner (*15*). Erk activation mediated by 5HT1A receptors, but not by angiotensin AT1A receptors, was inhibited by expression of either P91G-P121E β-arrestin 1 or V53D β-arrestin 1 (Fig. 6B). These data suggest that β-arrestin 1–dependent sequestration of GPCRs and recruitment of c-Src play a role in the initiation of Ras-dependent signals via a distinct subset of G protein–coupled receptors.

G protein–coupled receptors as scaffolds for the assembly of mitogenic signaling complexes. Several cell surface receptors that lack intrinsic tyrosine kinase activity, including antigen receptors on T and B cells, as well as the receptors for growth hormone, erythropoietin, and several cytokines, stimulate tyrosine phosphorylation through association with Src family kinases such as Src, Fyn, Yes, Lck, Hck, and Lyn (*25*). Our data suggest that "desensitized" β_2ARs function in an analogous manner, serving as scaffolds for the Src-dependent activation of Ras signaling pathways (Fig. 7). Agonist-dependent binding of β-arrestins to the receptor induces the formation of a multiprotein complex containing receptor, β-arrestin, and c-Src, which functions both to recruit activated Src kinase to the plasma membrane and to target the receptor-kinase complex to clathrin-coated pits. Both kinase recruitment and targeting are apparently required for β_2AR-mediated activation of the Erk pathway, because impairing either process inhibited the receptor-mediated activation of Erks.

Ras-dependent activation of Erks by β_2ARs and LPA receptors in COS-7 (*26*) and HEK-293 cells (*10*) requires the release of βγ subunits from G proteins. This may reflect the central role of βγ subunits in the regulation of receptor endocytosis. βγ subunits mediate agonist-induced membrane translocation of GRKs 2 and 3 (*27, 28*), which phosphorylate receptors and thereby increase their affinity for β-arrestins. In addition, βγ subunits bind to dynamin 1 and regulate its activity, and sequestration of βγ subunits directly inhibits clathrin-mediated endocytosis (*29*).

Inhibition of clathrin-mediated endocytosis by a dominant inhibitory mutant of dynamin blocks activation of Erks by EGF (*30*), suggesting that RTK-mediated signaling to Erks also involves endocytic trafficking. The binding of β-arrestins to GPCRs uncouples the receptor from its cognate G protein and

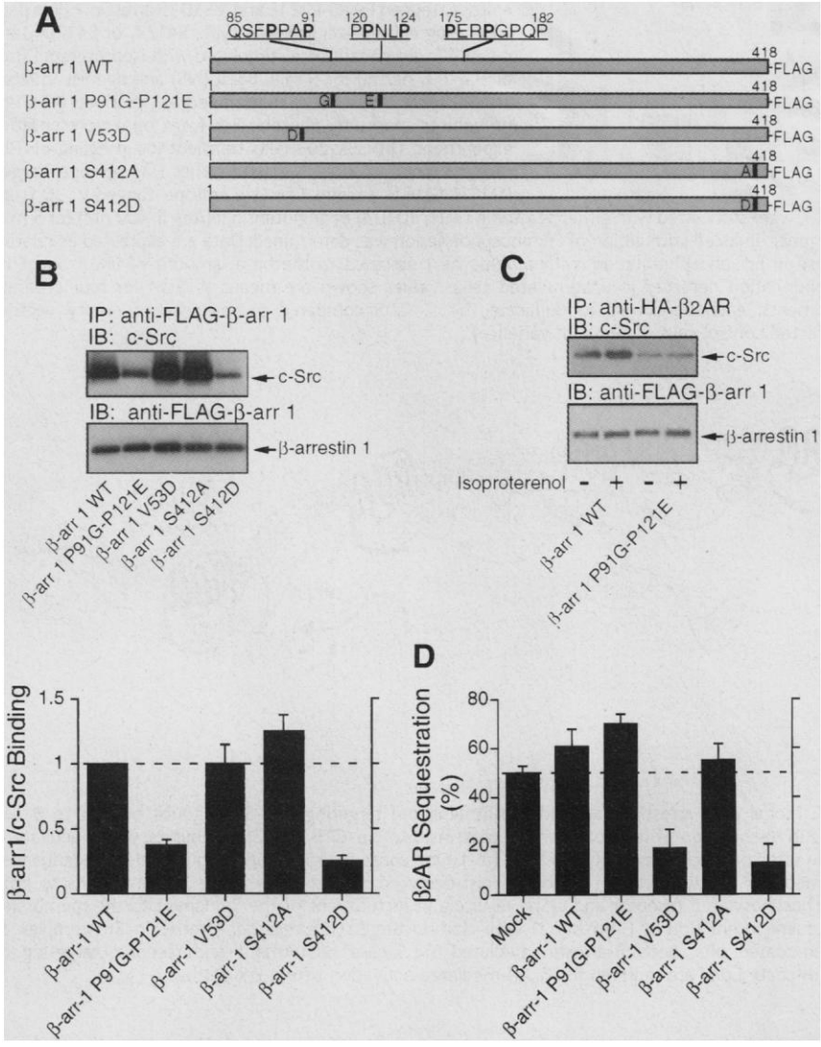

Fig. 5. Characterization of β-arrestin 1 mutants with respect to c-Src binding and β_2AR sequestration. (**A**) Schematic representation of wild-type and point mutations of Flag epitope–tagged β-arrestin 1. Abbreviations for amino acid residues: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; and S, Ser. (**B**) Effect of point mutations in β-arrestin 1 on its ability to coprecipitate c-Src. Upper panel: Anti-Flag immunoprecipitates were prepared from HEK-293 cells transiently overexpressing wild-type c-Src with Flag epitope–tagged β-arrestin 1 (WT), P91G-P121E β-arrestin 1, V53D β-arrestin 1, S412A β-arrestin 1, and S412D β-arrestin 1. Immunoprecipitated β-arrestins (lower immunoblot) and coprecipitated c-Src (upper immunoblot) are shown. Lower panel: Coprecipitated c-Src was quantified relative to the amount of wild-type and mutant β-arrestin 1 immunoprecipitated. Data are means ± SD of four independent experiments. (**C**) Formation of β_2AR–β-arrestin 1–c-Src complexes in COS-7 cells transiently overexpressing HA epitope–tagged β_2AR, wild-type or P91G-P121E β-arrestin 1, and wild-type c-Src. Transfected cells were incubated for 1 min in the absence (–) or presence (+) of isoproterenol before cross-linking and immunoprecipitation of HA epitope–tagged β_2ARs. Coprecipitated c-Src (upper immunoblot) and β-arrestins (lower immunoblot) were detected by protein immunoblotting. (**D**) Agonist-induced β_2AR sequestration in HEK-293 cells. Receptor sequestration induced by 30 min exposure to isoproterenol was determined by flow cytometry (*41*) performed on cells transiently overexpressing Flag epitope–tagged β_2AR without (Mock) or with Flag epitope–tagged wild-type β-arrestin 1 or β-arrestin 1 point mutations. Data are means ± SD of three independent experiments.

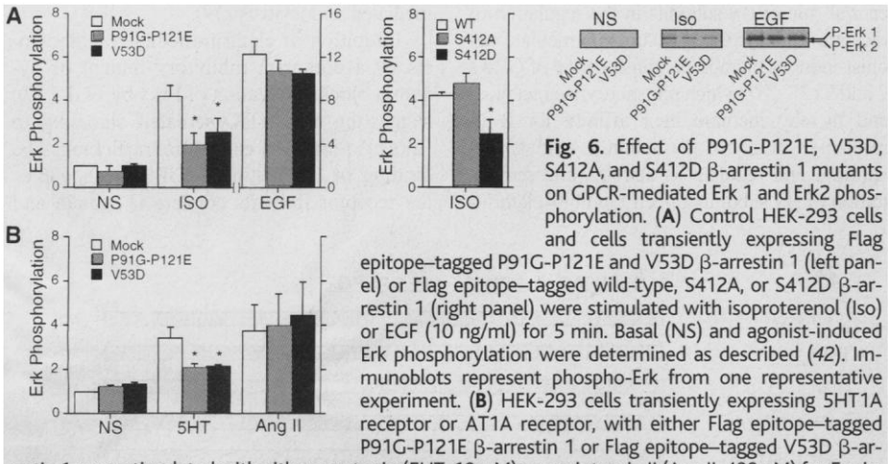

Fig. 6. Effect of P91G-P121E, V53D, S412A, and S412D β-arrestin 1 mutants on GPCR-mediated Erk 1 and Erk2 phosphorylation. (**A**) Control HEK-293 cells and cells transiently expressing Flag epitope–tagged P91G-P121E and V53D β-arrestin 1 (left panel) or Flag epitope–tagged wild-type, S412A, or S412D β-arrestin 1 (right panel) were stimulated with isoproterenol (Iso) or EGF (10 ng/ml) for 5 min. Basal (NS) and agonist-induced Erk phosphorylation were determined as described (*42*). Immunoblots represent phospho-Erk from one representative experiment. (**B**) HEK-293 cells transiently expressing 5HT1A receptor or AT1A receptor, with either Flag epitope–tagged P91G-P121E β-arrestin 1 or Flag epitope–tagged V53D β-arrestin 1, were stimulated with either serotonin (5HT, 10 μM) or angiotensin II (Ang II, 400 nM) for 5 min, and agonist-induced stimulation of Erk phosphorylation was determined. Data are expressed as relative increase in Erk phosphorylation, with a value of 1 assigned to the basal amount of Erk 1 and Erk2 phosphorylation detected in unstimulated cells. Values shown are means ± SEM for four separate experiments, each performed in duplicate. $*P < 0.05$ compared to response of empty vector–transfected control cells (analysis of variance).

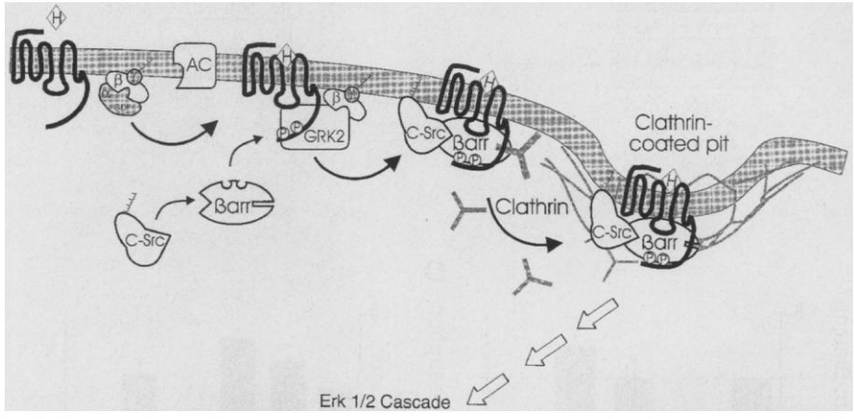

Fig. 7. Model of β-arrestin–mediated recruitment and targeting of c-Src. Agonist binding to β_2ARs results in dissociation of heterotrimeric G proteins into Gα-GTP and Gβγ subunits, which activate G protein effectors such as adenylyl cyclases (AC). One consequence of Gβγ subunit release is enhanced GRK-mediated phosphorylation of the agonist-occupied receptor. β-Arrestin 1 (βarr) binds to both GRK-phosphorylated receptor and c-Src, resulting in recruitment of the Src kinase to the membrane. Subsequent interaction of β-arrestin 1 with clathrin targets the receptor–β-arrestin–Src complex to clathrin-coated pits. Both β-arrestin–mediated Src kinase recruitment and receptor targeting to clathrin-coated pits are required for β_2AR-mediated activation of the Erk pathway.

mediates its translocation to the clathrin-coated pit (*2, 3*). Our data indicate that β-arrestins also function as adapter proteins that link GPCRs to tyrosine kinase–dependent growth regulatory pathways. Association of Src with the NH_2-terminus of β-arrestin 1 provides the structural basis for agonist-dependent recruitment of the tyrosine kinase to the receptor. Thus, β-arrestin binding and receptor internalization, processes that terminate G protein activation, apparently also represent critical events for the initiation of mitogenic signals from the GPCR.

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20. Immune complexes containing either whole-cell Src kinase or β-arrestin 1–associated Src were incubated for 15 min at 20°C in 30 μl of reaction mix [10 mM Pipes (pH 7.0), 10 mM $MnCl_2$, 5 mM GST-GAP p62 Src kinase substrate, 10 μM adenosine triphosphate (ATP), and 10 μCi of [γ-^{32}P]ATP]. Reactions were terminated by the addition of 2× Laemmli sample buffer, and phosphorylated GST-GAP p62 was resolved by SDS–polyacrylamide gel electrophoresis (PAGE). ^{32}P incorporation was determined with a Storm Phosphorimager.
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35. Transient transfection of HEK-293 cells was performed using a modified calcium phosphate precipitation method (*31*). COS-7 cells were transiently transfected with Lipofectamine (*9*). Monolayers of transfected cells were incubated in serum-free medium for 16 to 20 hours before stimulation (*9*).
36. Confocal microscopy was performed on a Zeiss LSM-410 laser scanning microscope using a Zeiss 40× oil immersion lens (numerical aperture 1.3). Fluorescent signals were collected with the Zeiss LSM software in the photon-counting mode using single-line excitation (*27*). Colocalization studies of fluorescein-labeled β_2AR and Texas Red–labeled c-Src fluorescence were done with dual excitation (488 and 568 nm) and emission (515 to 540 nm, fluorescein; 590 to 610 nm, Texas Red) filter sets. Specificity of labeling and absence of signal crossover were established by examination of single-labeled samples.
37. Cells in 100-mm dishes were stimulated at 37°C in phosphate-buffered saline (PBS) containing 10 mM Hepes (pH 7.4), as described in the figure legends. Stimulations were terminated by the addition of dithiobis[succinimidylpropionate] (DSP) to a final concentration of 2 mM, and plates were rocked for 30 min at room temperature. Cells were washed three times by centrifugation at 4°C with PBS/Hepes to

remove unreacted DSP, then lysed in RIPA buffer (*10*) before immunoprecipitation.

38. Immunoprecipitation and immunoblotting of Flag and HA epitope–tagged β_2ARs, HA epitope–tagged AT1A receptors, Flag epitope–tagged β-arrestins, and c-Src were performed with commercially available antisera as described (*9*, *31*). Immunoprecipitation and immunoblotting of His^6-tagged β-arrestin 1 were performed with a rabbit polyclonal antiserum raised against a GST–β-arrestin 1 COOH-terminus fusion protein (*32*). Immune complexes on nitrocellulose were visualized by enzyme-linked chemiluminescence and quantified by scanning laser densitometry. The stoichiometry of β-arrestin 1 to c-Src in receptor immunoprecipitates was determined by normalizing immunoblot intensities to the signal obtained from known amounts of purified recombinant His^6-tagged β-arrestin 1 and c-Src resolved along with the receptor immunoprecipitates.
39. Flag epitope–tagged truncation or deletion mutants of β-arrestin 1 were prepared by the polymerase chain reaction (PCR), incorporating an Eco RI site, minimal Kozak sequence (ACC), and initiator methionine codon into the 5′ primer, and the Flag epitope sequence, stop codon, and an Xho I site into the 3′ primer. PCR products were subcloned into a peptide minigene expression cassette as described (*33*). DNA sequences were confirmed by dideoxynucleotide sequencing.
40. Recombinant His^6-tagged β-arrestin 1 and recombinant human c-Src or c-Src SH2 or SH3 domain GST fusion proteins were combined for 20 min at room temperature in 20 μl of 10 mM Pipes (pH 7.0) before samples were diluted with 0.5 ml RIPA buffer, and His^6-tagged β-arrestin 1 was immunoprecipitated using rabbit polyclonal anti-His^6 immunoglobulin G (IgG). After resolution by SDS-PAGE, both the His^6-tagged β-arrestin 1 and coprecipitated Src-GST fusion proteins were detected simultaneously by protein immunoblotting with rabbit polyclonal anti-GST–β-arrestin 1. Competition for binding between c-Src and GST–Src SH2 or GST–Src SH3 was performed with the GST-Src fusion protein in 20-fold excess.
41. 12CA5 epitope–tagged β_2ARs (500 to 1000 fmol/mg whole-cell protein) expressed in HEK-293 cells in six-well plates were treated with or without isoproterenol for 30 min in serum-free medium at 37°C. Cell surface receptors were labeled with 12CA5 mAb, using fluorescein isothiocyanate–conjugated goat antibody to mouse IgG as secondary antibody. Receptor sequestration was quantified as loss of cell surface receptors, as measured by flow cytometry (*34*). Receptor expression was determined by saturation binding of [^{125}I]pindolol.
42. Aliquots of whole-cell lysate from appropriately stimulated cells (30 μg of protein per lane) were resolved by SDS-PAGE, and Erk 1 and Erk2 phosphorylation was detected by protein immunoblotting with rabbit polyclonal phospho-MAP kinase–specific IgG. Quantitation of Erk 1 and Erk2 phosphorylation was performed with a Storm Phosphorimager. After quantitation of Erk 1 and Erk2 phosphorylation, nitrocellulose membranes were stripped of Ig and reprobed with rabbit polyclonal anti-Erk 2 IgG to confirm equal loading of Erk protein.
43. R.J.L. and M.G.C. are investigators with the Howard Hughes Medical Institute. Supported in part by NIH grants DK02352 and DK55524 (L.M.L), HL16037 (R.J.L), NS19576 (M.G.C.), and Heart and Stroke Foundation of Ontario Grant NA3349 (S.S.G.F.). G.J.D.R. is supported by NIH Medical Scientist Training Program Grant T32GM-07171. We thank M. J. Eck for purified recombinant c-Src and D. Addison and M. Holben for excellent secretarial assistance.

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"Dip-Pen" Nanolithography

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A direct-write "dip-pen" nanolithography (DPN) has been developed to deliver collections of molecules in a positive printing mode. An atomic force microscope (AFM) tip is used to write alkanethiols with 30-nanometer linewidth resolution on a gold thin film in a manner analogous to that of a dip pen. Molecules are delivered from the AFM tip to a solid substrate of interest via capillary transport, making DPN a potentially useful tool for creating and functionalizing nanoscale devices.

Lithographic methods are at the heart of modern-day microfabrication, nanotechnology, and molecular electronics. These methods often rely on patterning of a resistive film, followed by a chemical etch of the substrate. Dip-pen technology, in which ink on a sharp object is transported to a paper substrate via capillary forces, is approximately 4000 years old (*1*) and has been used extensively throughout history to transport molecules on macroscale dimensions. Here we report experiments that merge these two related but, with regard to scale and transport mechanism, disparate concepts to develop a new type of dip-pen nanolithography (DPN). DPN uses an atomic force microscope (AFM) tip as a "nib," a solid-state substrate (in this case, Au) as "paper," and molecules with a chemical affinity for the solid-state substrate as "ink." Capillary transport of molecules from the AFM tip to the solid substrate is used in DPN to directly "write" patterns consisting of a relatively small collection of molecules in submicrometer dimensions.

DPN is not the only lithographic method that allows one to directly transport molecules to substrates of interest in a positive printing mode. For example, microcontact printing, which uses an elastomer stamp, can deposit patterns of thiol-functionalized molecules directly onto Au substrates (*2*–*6*). This method is a parallel technique, allowing one to deposit an entire pattern or series of patterns on a substrate of interest in one step (*2*–*19*), which is an advantage over a serial technique such as DPN unless one is trying to selectively place different types of molecules at specific sites within a particular type of nanostructure. In this regard, DPN complements microcontact printing and many other existing methods of micro- and nanofabrication (*2*–*19*). Finally, there are a variety of negative printing techniques that rely on scanning probe instruments, electron beams, or molecular beams to pattern substrates, using self-assembled monolayers (SAMs) and other organic materials as resist layers (*7*–*19*), that is, to remove material for subsequent processing or adsorption steps. However, DPN can deliver relatively small amounts of a molecular substance to a substrate in a nanolithographic fashion that does not rely on a resist, a stamp, complicated processing methods, or sophisti-

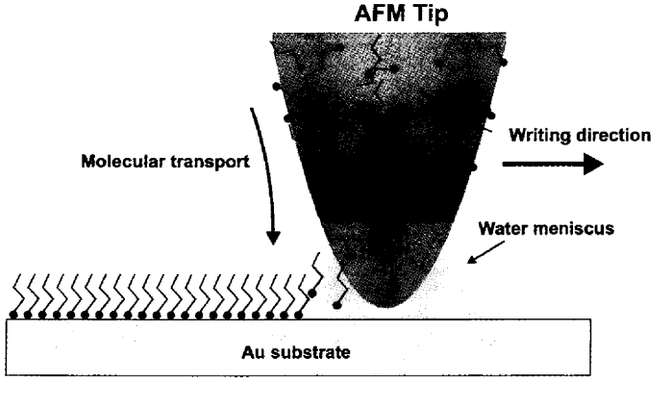

Fig. 1. Schematic representation of DPN. A water meniscus forms between the AFM tip coated with ODT and the Au substrate. The size of the meniscus, which is controlled by relative humidity, affects the ODT transport rate, the effective tip-substrate contact area, and DPN resolution.

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