pp60Src Mediates Insulin-stimulated Sequestration of the β_2 -Adrenergic Receptor: Insulin Stimulates pp60Src Phosphorylation and Activation

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Insulin stimulates a rapid phosphorylation and sequestration of the β_2 -adrenergic receptor. Analysis of the signaling downstream of the insulin receptor with enzyme inhibitors revealed roles for both phosphatidylinositol 3-kinase and pp60Src. Inhibition of Src with PP2, like the inhibition of phosphatidylinositol 3-kinase with LY294002 [2-(4-morpholynyl)-8-phenyl-4H-1-benzopyran-4-one], blocked the activation of Src as well as insulin-stimulated sequestration of the β_2 -adrenergic receptor. Depletion of Src with antisense morpholinos also suppressed insulin-stimulated receptor sequestration. Src is shown to be phosphorylated/activated in response to insulin in human epidermoid carcinoma A431 cells as well as in mouse 3T3-L1 adipocytes and their derivative 3T3-F422A cells, well-known models of insulin signaling. Inhibition of Src with PP2 blocks the ability of insulin to sequester β_2 -adrenergic receptors and the translocation of the GLUT4 glucose transporters. Insulin stimulates Src to associate with the β_2 -adrenergic receptor/AKAP250/protein kinase A/protein kinase C signaling complex. We report a novel positioning of Src, mediating signals from insulin to phosphatidylinositol 3-kinase and to β_2 -adrenergic receptor trafficking.

INTRODUCTION

β_2 -Adrenergic receptors (β_2 ARs) are members of the superfamily of G protein-coupled receptors (GPCRs) and display desensitization in response to β_2 -adrenergic agonists as well as counterregulation by several growth factor receptors with intrinsic tyrosine kinase activity (Morris and Malbon, 1999). Sequestration of the β_2 AR plays a central role in agonist-induced and insulin-induced regulation of β -adrenergic signaling (Lefkowitz, 1998; Morris and Malbon, 1999). β_2 ARs are sequestered in response to insulin and this loss of the surface complement of receptors plays a critical role in the counterregulatory physiological effects of insulin on catecholamine action (Karoor *et al.*, 1998). Although information has been gained on agonist-induced trafficking of GPCRs (Carman and Benovic, 1998; Gagnon *et al.*, 1998), much less

is known about how counterregulation by tyrosine kinases receptors influences GPCR trafficking.

β_2 ARs are substrates for insulin-stimulated tyrosine phosphorylation (Karoor and Malbon, 1998). In vivo, insulin stimulates the phosphorylation on two major tyrosine residues, Y350 and Y364, both residues located in the C-terminal cytoplasmic domain of the β_2 AR (Karoor *et al.*, 1995). Phosphorylation of the Y350 residue creates an SH2-binding site to which several molecules can dock, including Grb2, the p85 catalytic domain of phosphatidylinositol 3-kinase, and the GTPase dynamin (Shih and Malbon, 1998). In vitro, insulin stimulates the purified insulin receptor to phosphorylate recombinant β_2 -adrenergic receptor on these same residues (Baltensperger *et al.*, 1996; Doronin *et al.*, 2000). The phosphorylation of the β_2 -adrenergic receptor impairs its function, a blockade that requires Grb2 with an intact SH2 domain (Shih and Malbon, 1998). Although inducing a rapid, profound sequestration of the β_2 AR, β -adrenergic agonists and insulin display differences in the pathways by which the sequestration occurs (Karoor *et al.*, 1998). The sequestration in response to insulin, but not β -adrenergic agonist, can be blocked with inhibitors of phosphatidylinositol 3-kinase (PI3K), such as wortmannin or LY294002 [2-(4-morpholynyl)-8-phenyl-4H-1-benzopyran-4-one] (Wang *et al.*, 2000).

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Abbreviations used: pp60Src, phosphorylated, activated phosphoprotein 60-M, Src; p60Src, phosphoprotein 60-M, Src; and β_2 AR, β_2 -adrenergic receptor.

Recently, the nonreceptor tyrosine kinase Src has been shown to be involved in β -adrenergic agonist-induced desensitization (Luttrell *et al.*, 1996), associating with the β_2 AR and the scaffold protein gravin, also known as AKAP250 (Fan *et al.*, 2001a,b), leading to its phosphorylation and activation of G protein receptor kinases (Ruiz-Gomez and Mayor, 1997; Sarnago *et al.*, 1999). Whether Src functions in insulin-stimulated counterregulation of the β_2 AR is not known. Investigation of the role of Src in this insulin-stimulated response is the focus of the current studies. Herein, we report that insulin activates Src, mediating signaling from the insulin receptor to the level of PI3-kinase activation and the trafficking of the β_2 AR.

MATERIALS AND METHODS

Materials

The plasmid encoding the enhanced green fluorescent protein (eGFP)-tagged human β_2 AR (in pCDNA3) was obtained from Dr. Jeffrey Benovic (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). The pCDNA3GFP-GLUT4 expression vector was a gift of Dr. Jeffrey Pessin (Department of Physiology and Biophysics, University of Iowa, Iowa City, IA), and the expression vector harboring the constitutively active Src Y527F mutant (CA-Src) was generously provided by Dr. Joan Brugge (Department of Cell Biology, Harvard Medical School, Boston, MA). To analyze and characterize an expression of green fluorescent protein (GFP)-tagged β_2 AR, the following antibodies were used: anti- β_2 AR (CM02, antipeptide antibody to the exofacial domain of the β_2 AR; Wang *et al.*, 1989a) and anti-GFP rabbit polyclonal antibodies (Quantum Biotechnologies, Montreal, Quebec, Canada); goat anti-rabbit antibody conjugated with horseradish peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, MD); and mouse anti-insulin receptor β subunit (p95; Transduction Laboratories, Lexington, KY).

Cell Culture

Human epidermoid carcinoma A431 wild-type cells and A431 clones stably expressing either eGFP-tagged β_2 AR or eGFP-tagged GLUT4 glucose transporters were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin (George *et al.*, 1988; Wang *et al.*, 1991; Shih and Malbon, 1994, 1996; Shih *et al.*, 1999). The mouse 3T3-L1 and 3T3-F442A cells (American Type Culture Collection, Manassas, VA) were cultured in the same medium with an addition of $1\times$ nonessential amino acids (Invitrogen, Carlsbad, CA) and were induced to the adipocyte phenotype by the treatment with dexamethasone and 1-methyl-3-isobutylxanthine, as described previously (Green and Kehinde, 1975; Wang *et al.*, 1992). Before analysis for insulin action, A431, 3T3-L1, and 3T3-F442 cells were serum starved overnight (Karoor *et al.*, 1995).

Assay of Phosphorylation of Src

A431 cells were stimulated with 100 nM insulin for the indicated times, collected, and then lysed in a lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 40 mM sodium pyrophosphate, 50 mM KH_2PO_4 , 10 mM Na-molybdate, 2 mM Na-orthovanadate, 20 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.5% NP-40, 6 mM dithiothreitol, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 0.2 mM phenylmethylsulfonyl fluoride). Samples (50 μ g of protein/lane) were subjected to electrophoresis on 10% polyacrylamide gels, the separated proteins transferred to nitrocellulose, and the blots stained with antibodies, as described previously (Jho *et al.*, 1997). For these experiments, the blots were stained with rabbit anti-phospho-Src (Y416) antibodies purchased from Upstate Biotechnology (Lake Placid, NY). These antibodies specifically recognize only the Y416 phosphorylated, activated form of Src family members. Other antibodies

used for immunoblotting of samples of cell lysates were anti-Src, anti-Fyn, anti-Lck, anti-Lyn, and anti-Yes, each obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

Inhibitor and Morpholino Antisense Oligomer Studies

A431 cells stably expressing eGFP-tagged β_2 -adrenergic receptors were pretreated for 30 min with one of the following inhibitors: the PI3K inhibitor LY294002 (20 μ M), the mitogen-activated protein kinase (MEK) inhibitor PD98059 (2'-amino-3'-methoxyflavone) ($< \mu$ M), or the inhibitor of nonreceptor Src family kinases PP2 (50 nM). After pretreatment with an inhibitor, cells were incubated with or without 100 nM insulin for 30 min followed by live imaging of receptor localization. The morpholino antisense oligomers were designed and synthesized by Gene Tools (Philomath, OR). The antisense sequence selected for Src was 5'-ATGGGTAGCAACAA-GAGCAAGCC-3', with the corresponding sense sequence used as a control. Cells were treated according to the protocol provided by Gene Tools. Treatment with the morpholinos was limited to 48 h, because extending the period of treatment to >72 h resulted in significant cell death.

Src Activity Assay

Whole-cell lysates were prepared from either A431 or 3T3-L1 clones. The phosphotransferase activity of Src kinase was determined in immunoprecipitates from cell lysates by using Src assay kit (Upstate Biotechnology), following the manufacturer's instructions. The preferred Src substrate peptide KVEKIGEGTYGVVYK corresponding to amino acids 6–20 of p34^{cdc2} was used in these studies. The assay is linear for incubation times to 30 min. Samples were assayed in quadruplicate and the amount of incorporated label determined by scintillation counting.

PI3K Activity Assay

Crude cell membrane fractions were resuspended in a reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 1 mM EGTA. An aliquot of membrane (100 μ g/50 μ l) was added to a reaction mixture containing 13 mM MnCl_2 , 65 μ M phosphatidylinositol diphosphate, 0.2 μ g/ μ l phosphatidylserine, and 1 mM ATP (containing 1 μ Ci of [γ^{32} P]ATP). The reaction was initiated by addition of the protein sample and maintained at 30°C for 20 min. The reaction was stopped with 2 N HCl and then an aliquot (160 μ l) of chloroform:methanol (1:1) mixture was added. The samples were subjected to centrifugation at $10,000 \times g$ for 1 min and the lower phase was transferred to a new tube and washed by chloroform:methanol:0.1 N HCl (1:1:1) twice. The lower phase was collected and dried under vacuum. The samples were resuspended in 10 μ l of chloroform:methanol (95:5) and then spotted onto the origin of a thin layer chromatography (TLC) plate (Song *et al.*, 2001). The plate was developed in chloroform:methanol:25% $\text{NH}_4\text{OH}:\text{H}_2\text{O}$ (100:70:25:15). The resolved plate was dried and exposed to films Eastman Kodak, Rochester, NY) or PhosphorImager (Molecular Dynamics, Sunnyvale, CA) cassette.

Radioligand Binding Studies

The number of β_2 ARs was determined by radioligand binding by using the high-affinity, β -adrenergic antagonist [125 I]iodocyanopindolol (ICYP). Intact A431 cells were incubated with 0.5 nM ICYP (PerkinElmer Life Sciences, Boston, MA) in the presence or absence of 10 μ M propranolol at 23°C for 90 min. The incubation buffer contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl_2 , and 150 mM NaCl. The cells were collected under vacuum on GF/C membranes (Whatman, Maidstone, United Kingdom) and washed rapidly three times. The amount of ICYP bound to the washed cell mass retained by the filter was quantified by use of gamma counting (George *et al.*, 1988).

Figure 1. Insulin counterregulates catecholamine action by sequestration of β_2 ARs. A431 cells stably transfected with an expression vector harboring the cDNA for the human β_2 AR tagged with enhance GFP were used. (A) Immunoblots of WT cells and clones expressing β_2 AR-GFP were stained with antibodies to either β_2 AR or GFP. (B) Fluorescent images of live A431 cells that express β_2 AR-GFP were collected by using epifluorescence (inverted) microscopy after treatment of the cells with either the β -adrenergic agonist isoproterenol (10 μ M, +Iso) or insulin (100 nM, +Ins) for 30 min (bottom row). White arrows highlight receptors localized to the cell membrane; yellow arrowheads indicate the receptors in perinuclear zone. The same experiments were performed, except that in this case the cells were fixed and the images were acquired by confocal microscopy (top row). (C) Time course of β_2 AR-GFP translocation after A431 cell stimulation with insulin. A431 cells were stimulated with 100 nM insulin and imaged live on the charge-coupled device camera as repetitive frames were collected from the same spot over the time period of 35 min.

Epifluorescence of Receptor/GLUT4 Translocation (Live Cell Microscopy)

Clones expressing either the eGFP-tagged β_2 AR or the eGFP-tagged GLUT4 were seeded onto coverslips of either four- or eight-chamber

slides. Confluent cells were serum deprived overnight, treated with the drugs, and carefully washed three times with HBB buffer (1 $\times$ Hanks' balanced salt solution, 0.1% bovine serum albumin, 10 mM HEPES, pH 7.5; Invitrogen). Fresh DMEM medium without phenol red and serum was added and cells were imaged rapidly in an

Figure 2.

inverted fluorescent microscope (Nikon, Tokyo, Japan), fitted with a 40 $\times$ objective (oil immersion). An acquisition of digital images was performed using a cooled charge-coupled device Princeton camera and WinView software (Roper Scientific, Inc., Trenton, NJ).

Confocal Microscopy

Clones were grown on glass slides, treated with drugs as indicated, fixed with 2% paraformaldehyde in phosphate-buffer saline, washed, and then treated with SlowFade Antifade kit (Molecular Probes, Eugene, OR) to prevent photobleaching. The confocal microscopy was performed on Eclipse E600 microscope (Nikon) using argon laser (488 nm). Images acquired with C-imaging system (Compix, Cranberry Township, PA) operating SimplePCI software.

Data Presentation and Analysis

Unless otherwise noted, the values presented are mean values $\pm$ SEM and are representative of multiple (at least three), independent experiments. Adobe Photoshop5.5 and Illustrator 8.0 (Adobe Systems, Mountain View, CA) were used to prepare final images and figures.

RESULTS

To facilitate study of β_2 AR sequestration in response to insulin, we stably transfected human epidermoid carcinoma A431 cells, a well-characterized model of β_2 -adrenergic receptor biology (Delavie-Klutchko *et al.*, 1984; Kassiss *et al.*, 1987; Lohse *et al.*, 1990), with an expression vector harboring eGFP-tagged human β_2 AR (Fan *et al.*, 2001a). Stable clones were selected that expressed sufficient amounts of a GFP-tagged version of the human β_2 -adrenergic receptor (β_2 AR-GFP) to enable high-quality epifluorescence microscopy, but contributed <10% to the endogenous level of the receptor (Figure 1). Immunoblotting of wild-type (WT) and A431 clones (β_2 AR-GFP) stained with anti- β_2 AR antibodies revealed the endogenous (M_r of 65 kDa) receptor in the WT cells as well as the endogenous and the GFP-tagged versions (M_r of $\sim$ 95 kDa) in the A431 stable transfectants (Figure 1A).

Figure 2 (facing page). Inhibitors of PI3-kinase, inhibitors of Src phosphotyrosine kinases, and depletion of Src block insulin-induced sequestration of β_2 AR. (A) A431 clones were untreated (control) or treated with the PI3-kinase inhibitor LY294002, Src kinase inhibitor PP2, or the MEK inhibitor PD98059 and then stimulated with either isoproterenol (10 μ M, +Iso) or insulin (100 nM, +Ins). After 30 min with the hormones, the cells were examined by epifluorescence microscopy. White arrows highlight cell membrane-localized receptors; yellow arrowheads highlight perinuclear-localized receptors, sequestered from the surface. (B) A431 cells were treated with morpholinos antisense to Src for 48 h to deplete cellular levels of Src as well as sense morpholinos as a control. Cells were then stimulated either with 100 nM insulin (+Ins) or without insulin (control). After 30 min with the hormones, the cells were examined by epifluorescence microscopy. White arrows highlight cell membrane-localized receptors; yellow arrowheads highlight perinuclear-localized receptors, sequestered from the surface. (C) Immunoblot of total cell lysates of A431 cells that were untreated (control) or treated with morpholinos either antisense (antisense) or sense (sense) to Src. The lysates were subjected to SDS-PAGE, the resolved proteins transferred to nitrocellulose blots, and stained with an anti-Src antibody. The blots were stained with antibody against the insulin receptor β -subunit (p95 IR β) to establish equivalence of sample loading.

Staining of the blots with anti-GFP antibodies, in contrast, identified only the 95 kDa- M_r , GFP-tagged species in the A431 clones and identified no immunoreactivity in blots from the WT cells. Radioligand binding with the radiolabeled, high-affinity β -adrenergic antagonist [125 I]iodocyanopindolol, revealed $\sim$ 35,000 receptors/WT A431 cell and $\sim$ 38,000 receptors/A431-transfected cell (our unpublished data). The GFP-tagged human β_2 AR has been characterized fully and displays activation, desensitization, and sequestration in response to agonist, as does the native β_2 AR (Kallal *et al.*, 1998; Fan *et al.*, 2001b).

We made use of both epifluorescence and confocal microscopy to examine the effects of insulin on the localization of the β_2 AR (Figure 1B). Treatment with insulin (100 nM, +Ins) provoked a substantial sequestration of β_2 AR to perinuclear regions of the cell (yellow arrowheads) away from the cell membrane (white arrows), within 15 min of the addition of insulin. Challenge with the β_2 -adrenergic agonist isoproterenol (10 μ M, +Iso) was examined as a control. β_2 ARs were observed to sequester in the same manner in response to stimulation by either insulin or by isoproterenol. The results of confocal microscopy were in agreement with the epifluorescence data, revealing a significant translocation of β_2 AR from the cell membrane to a perinuclear locale in response to homologous (agonist) and heterologous (insulin) stimulation. A time course of β_2 AR localization in response to 100 nM insulin extended to 35 min revealed the profound redistribution of β_2 AR stimulated by insulin (Figure 1C). In the absence of insulin, the majority of the β_2 AR is localized to the cell membrane. Within 30–35 min of insulin treatment the bulk the receptor is now found densely packed within a perinuclear region of the cell. Washout of the insulin was followed by a full recovery of the β_2 AR to the cell membrane within 60 min, trafficking back from the perinuclear region of the cell (our unpublished data). β_2 ARs display a high-level of recycling after internalization, with little discernable degradation over the period of a few hours (Wang *et al.*, 1989b). Significant degradation (20–30%) of β_2 AR is only detected after 24 h of chronic agonist treatment (Shenoy *et al.*, 2001).

To unravel the details of the downstream signaling from insulin to the sequestration of β_2 ARs, we first investigated roles of three likely signaling enzymes: PI3-kinase, the Src family of nonreceptor tyrosine kinases, and the mitogen-activated protein kinase cascade. Using selective inhibitors of each enzyme, we investigated whether inhibition would promote blockade of insulin-stimulated sequestration of β_2 ARs (Figure 2A). A431 clones were treated with insulin and the sequestration of GFP-tagged β_2 ARs examined by epifluorescence microscopy at 30 min postchallenge. The PI3-kinase inhibitor LY294002 effectively blocked the sequestration of β_2 AR induced by insulin. The ability of isoproterenol to induce the agonist-specific sequestration of β_2 AR, unlike insulin action, was unaffected by inhibition of PI3-kinase. Treatment with the PD98059 inhibitor of MEK in the mitogen-activated protein kinase pathway, in contrast to the effects of LY294002, did not alter the ability of insulin to sequester β_2 AR. Previously, it was shown that the nonreceptor tyrosine kinase Src plays a role in agonist-specific desensitization and internalization of β_2 ARs, so the effects of the PP2 inhibitor of Src family kinases were tested on insulin-induced sequestration of β_2 ARs. We found that PP2 was

Figure 3. Either LY294002 or Src inhibitor PP2 blocks activation of PI3-kinase by insulin. A431 cells were untreated (–), treated with LY294002 (LY), or treated with the Src inhibitor PP2 (PP2), and then stimulated with 100 nM insulin for 30 min. (A) PI3-kinase activity was determined in the cells by measurement of its product phosphatidylinositol(3,4,5)P3 by using TLC. A representative experiment is shown. (B) Summation of the effects of LY294002 and PP2 on insulin stimulated PI3-kinase activity, displayed as arbitrary units with the control set as “1.” The results are mean values from three separate experiments.

a potent inhibitor of insulin-stimulated sequestration of β_2 ARs (Figure 2A). These results piqued our interest in further exploring the seemingly obligate role of Src in this action of insulin.

Based upon the effects of the Src family kinase inhibitor, we explored the effects of the depletion of Src on the ability of insulin to provoke the sequestration of β_2 ARs in A431 cells (Figure 2B). Depletion of Src was accomplished using morpholino oligomers (morpholinos) antisense to Src, and sense morpholinos were used as a control. Treating the cells with antisense morpholinos for 48 h resulted in a loss of insulin-stimulated sequestration of β_2 AR, whereas treatment with sense morpholinos did not (Figure 2B). Immunoblots of whole-cell lysates stained with anti-Src antibodies demonstrate a depletion of Src (Figure 2C). Attempts to prolong the exposure to the antisense morpholinos resulted in cell death, suggesting that some level of Src expression is required for A431 cell viability. Parallel experiments performed with thioate(S)-modified oligodeoxynucleotides antisense to Src provided comparable data to the morpholinos (our unpublished data).

The activation of PI3-kinase is an early and essential response in the insulin-signaling cascade, so we explored whether Src kinase acts upstream or downstream of PI3-kinase (Figure 3). A431 cells were treated with insulin and the activation of PI3-kinase examined by assay of its product PI(3,4,5)P3 by using TLC. Insulin stimulated a rapid and robust increase in PI3-kinase activity, as shown in a representative chromatogram (Figure 3A). Analysis from replicate experiments revealed a two- to threefold increase in PI3-kinase activity in response to stimulation with 100 nM insulin in these cells (Figure 3B). As a control, we examined

Figure 4. Insulin stimulates activation of Src. A431 cells were stimulated with insulin and the activation of Src determined by use of activation-specific antibodies for Src (pp60Src; A and B) or by analysis of Src activity by using a preferred substrate peptide (KVEKIGEGTYGVVYK; C). (A) Cells were stimulated with 100 nM insulin and the time course of Src activation was measured using activation-specific Src antibodies to stain immunoblots of samples from the whole-cell lysates. Loading equivalence was checked using an antibody that recognizes Src (p60Src). (B) Cells were stimulated with 25–100 nM insulin for 30 min and Src activation measured using activation-specific Src antibodies to stain immunoblots from the whole-cell lysates. Loading equivalence was checking using an antibody that recognizes Src (p60Src). (C) Cells were stimulated with 100 nM insulin and the time course of Src activation measured by assay of the phosphorylation of a Src preferred substrate by using Src immunoprecipitated with anti-Src protein A/G-agarose from whole-cell lysates. The results displayed are mean values of three separate experiments. (D) Cells were treated with either LY294002 or the Src kinase inhibitor PP2 and then stimulated with 100 nM insulin for 30 min. Src activation was measured by assay of the phosphorylation of Src preferred substrate by using Src immunoprecipitated from whole-cell lysates. The results displayed are mean values $\pm$ SEM of five separate experiments.

the effects of the addition of the PI3-kinase inhibitor LY294002, which abolished the ability of insulin to activate PI3-kinase. Remarkably, treating the cells with the Src family inhibitor PP2 proved equally effective as LY294002 in inhibiting insulin activation of PI3-kinase. These data suggest a

Figure 5. Inhibition of PI3-kinase and Src phosphotyrosine kinases blocks insulin-induced translocation of GLUT4 to the cell membrane. A431 clones stably transfected with an expression vector harboring the mouse eGFP-tagged GLUT4 were either untreated (control) or treated with the PI3-kinase inhibitor LY294002 or the Src kinase inhibitor PP2, and then either stimulated with insulin (100 nM, +Ins) or untreated (basal). After 30 min of insulin stimulation, the cells were washed, fixed, and analyzed by epifluorescence confocal microscopy. White arrows highlight cell membrane-localized GLUT4; yellow arrowheads highlight GLUT4 residing in the cytoplasm.

critical role of Src kinase activation either upstream or collateral with the activation of PI3-kinase.

In the absence of reports in the literature regarding Src activation in response to insulin, we examined the obvious tenet that insulin stimulates Src activation (Figure 4). Src activation first was measured using activation-specific antibodies that recognize only the Y416 phosphorylated form of pp60Src. A431 cells treated with 100 nM insulin displayed a rapid activation of Src, detectable within 10 min and peaking within 30 min (Figure 4A). This time course for Src activation is not unlike that observed for the activation of insulin-sensitive pathways, such as GLUT4 translocation and glycogen synthase. Equivalent loading in these experiments was established by immunoblotting with antibodies to p60Src itself (Figure 4, A and B). The dose response for Src activation by insulin was investigated. Activation of Src was detected at the lowest concentration of insulin tested, 25 nM (Figure 4B). Maximal activation of Src occurred at concentrations of insulin from 50 to 100 nM, the same range of insulin concentration typically maximal for insulin-stimulated GLUT4 translocation and activation of glycogen synthase. Direct measurement of Src activity was performed using a preferred substrate, which likewise demonstrated activation of Src in response to insulin (Figure 4C). We next measured the Src activity in cells that were treated with either LY294002 or PP2. As predicted, treatment with PP2 inhibitor blocked insulin-stimulated activation of Src (Figure 4D). PP2 completely inhibits insulin-stimulated Src activity, but not the ambient levels of Src. The basis for this lack of inhibition of basal Src activity by PP2 is not clear. Treatment with LY294002, in sharp contrast, suppresses the ambient level of Src activity, but does not block Src activation. These data do demonstrate that activation of PI3-kinase is not obligate for insulin to activate Src, but that PI3-kinase seems necessary to sustain the ambient level of Src activity. Per-

haps association with some element of the β_2 AR signaling complex in the "basal", insulin-unstimulated state (Fan *et al.*, 2001a,b) may alter Src susceptibility of PP2 inhibition.

The GLUT4 hexose transporter is insulin sensitive; insulin stimulates its translocation from intracellular vesicles to the plasma membrane (Olefsky, 1999). In many respects, the sequestration of the β_2 AR from the cell membrane to perinuclear vesicles in response to insulin seems functionally to be the inverse of insulin-stimulated trafficking of GLUT4 transporters, a process that moves GLUT4 from the perinuclear vesicles to the cell membrane. To examine this intriguing relationship, A431 cells were transfected with a expression vector harboring an eGFP-tagged GLUT4 transporter and then the response to a challenge with insulin was examined by epifluorescence microscopy (Figure 5). In sharp contrast to the situation with the β_2 AR, the GLUT4 transporter is shown to reside in a perinuclear locale (see yellow arrowheads) in the absence of insulin and to be readily translocated to the cell membrane (white arrows) after a 30-min challenge with 100 nM insulin (Figure 5, a and d). Treating the cells with the PI3-kinase inhibitor LY294002 blocks the ability of insulin to translocate GLUT4 to the cell membrane (Figure 5, b and e), much as it blocks the ability of insulin to translocate the β_2 AR to the perinuclear space (Figure 2A). These data are consistent with the observations that inhibition of PI3-kinase in many cells blocks insulin action. Treatment with the Src inhibitor PP2 also blocks the ability of insulin to translocate GLUT4 to the cell membrane (Figure 5, c and f). Thus, in the context of the A431 cells both PI3-kinase and Src activation seem obligate for insulin action with respect to β_2 AR sequestration and GLUT4 translocation.

We extended these results obtained in A431 cells to a well-studied model of insulin action, the mouse 3T3-L1 adipocyte. The 3T3-L1 cells were induced to differentiate into adipocytes by treatment with dexamethasone and methyl-

sobutylxanthine (Green and Kehinde, 1975). The ability of insulin to stimulate activation of Src was measured using antibodies that recognize the activation-specific, Y416-phosphorylated form (pp60Src). The adipocyte cultures were stimulated with insulin and the whole-cell extracts were subjected to immunoblotting and staining with the anti-pp60Src antibody (Figure 6A). Sample loading was compared using immunoblotting and staining with antibodies specific for the p95 β -subunit of the insulin receptor. Insulin stimulated a dose-dependent activation of Src, with activation observed at the lowest concentration of insulin tested, 25 nM. The dose response with respect to insulin and Src activation in these adipocytes was comparable with that observed in the A431 cells (compare Figures 4B and 6A). Direct measurement of activity by using a Src-specific substrate was performed in 3T3-L1 cultures treated with 100 nM insulin (Figure 6B). The time course was similar to that observed in the A431 cells (compare Figures 4A and 6B). To further extend these studies, we used the mouse 3T3-L1 derivative F442A cell line. We were unable to obtain suitable levels of expression of GFP-tagged β_2 AR in the 3T3-L1 cells, but were successful using the F442A derivative for these same purposes. Mouse 3T3-F442A cells can be induced by various hormones to differentiate into adipocytes and have been used as a model for study of insulin action (Chen *et al.*, 1989). Cells were transfected with GFP-tagged β_2 AR and examined for the effects of insulin (Figure 6C). β_2 AR sequestration in response to insulin was similar to that observed in the A431 cells. Similarly, treatment with the Src inhibitor PP2 blocked the ability of insulin to sequester β_2 ARs in this model cell line of insulin action (compare Figures 2A and 6C). These data confirm the results obtained in the A431 cells, demonstrating not only insulin-stimulated Src activation but also insulin-stimulated sequestration of β_2 AR in well-studied models of insulin action.

GPCRs signal via complexes that include scaffold proteins (e.g., AKAP79 and AKAP250), protein kinase A, protein kinase C, protein phosphatases, and other molecules (Shih *et al.*, 1999; Dodge and Scott, 2000; Edwards and Scott, 2000; Fraser *et al.*, 2000; Lin *et al.*, 2000; Miller *et al.*, 2000; Cong *et al.*, 2001; Fan *et al.*, 2001a,b). Phosphorylation of the β_2 ARs in response to insulin at Y350 creates a docking site to which Grb2, the p85 regulatory subunit of PI3-kinase, dynamin, and Src may bind via SH2 domains (Shih and Malbon, 1996, 1998; Shih *et al.*, 1999). We sought to investigate the association of Src with the β_2 AR-signaling complex and ascertain its activation state, by using anti-pp60Src antibodies. A431 cells were either untreated (–), or treated with insulin (+Ins, 100 nM) for 30 min, lysed, and subjected to pull-down assays performed with antibodies to the β_2 ARs (Figure 7A). The β_2 AR complexes were then subjected to immunoblotting and staining for content of Src or pp60Src. The results demonstrate a sharp increase in the amount of Src associated with the β_2 AR complex (Figure 7A). Staining with activation-specific antibodies for pp60Src revealed activated Src accumulation with the β_2 AR complex in response to insulin. Staining with antibodies against β_2 AR established the equivalence of sample loading. Similar pull-down assays were performed with antibodies against Src (Figure 7B). Staining of the Src-associated complexes with anti- β_2 AR antibodies revealed the presence of β_2 ARs when the complexes were pulled down with antibodies against Src. The results ob-

Figure 6. Insulin stimulates activation of Src and sequestration of β_2 AR in mouse adipocytes in culture. Mouse 3T3-L1 cells were induced to differentiate into adipocytes. (A) Adipocyte cultures were stimulated with 25–200 nM insulin for 30 min and Src activation measured using activation-specific Src antibodies to stain immunoblots of samples from the whole-cell lysates. Loading equivalence was determined by using an antibody that recognizes insulin receptor β -subunit (p95 IR β) to stain the blots also. (B) Adipocyte cultures were stimulated with 100 nM insulin and the time course of Src activation measured by assay of the phosphorylation of a Src preferred substrate by using Src immunoprecipitated from whole-cell lysates. The results displayed are mean values of three separate experiments. (C) Mouse 3T3-F442 cells, a cell line derived from the 3T3-L1 cell line and also used as a model of insulin signaling, were transiently transfected with GFP-tagged β_2 ARs and used to study the effects of PP2 inhibitor on the ability of insulin (30 min, 100 nM, +Ins) to stimulate β_2 AR sequestration. The images shown are representative of at least three separate experiments.

tained with Src pull-downs from insulin-treated cells confirmed those obtained in the β_2 AR-targeted pull-downs (Figure 7, A and B). We next examined whether the insulin-

Figure 7. Insulin stimulates Src, but not Lyn, Fyn, or Yes, association with β_2 AR signaling complexes. A431 cells were stimulated without (-) or with 100 nM insulin (+Ins) for 30 min and whole-cell lysates were prepared and used for pull-down immunoprecipitations (IPs) with antibodies specific for either β_2 ARs (A and C) or Src (B). The immunoprecipitates were washed and subjected to SDS-PAGE and immunoblotting. The blots were stained with antibodies to one of the following proteins: activated Src (pp60Src); Src (p60Src); β_2 AR (β_2 AR); Lyn, Fyn, or Yes. A representative blot is shown for each.

stimulated increase in Src association with β_2 AR complexes was specific for Src or would be manifest for other members of the Src family. The results of these β_2 AR-targeted pull-down assays reveal that Src, but not Lyn (both 53- and 56-kDa M_r splice variants), Fyn, and Yes, shows insulin-induced increase in association of Src with β_2 AR signaling complexes (Figure 7C). Association of inactive Src with signaling complexes, including those containing the β_2 AR (Fan *et al.*, 2001a,b) and α IIb β 3 integrins (Oberfell *et al.*, 2002), provides an explanation for the association of Src with β_2 AR, although not activated (Figure 7C). Insulin is shown to stimulate both a sharp increase in Src association with β_2 AR (Figure 7C) and more importantly a sharp increase in Src activation (Figure 7A).

These studies demonstrate an obligate role of Src in the counterregulatory effects of insulin on β_2 AR action, exerted at the level of β_2 AR sequestration. In both the A431 cells and the 3T3-L1 adipocytes, we observed the ability of insulin to translocate Src to β_2 AR signaling complexes and to activate Src. To further probe the linkage between Src activation and β_2 AR sequestration, we first transfected A431 cells with an expression vector that harbors a constitutively active Y527F mutant form of chicken Src (CA-Src). Clones stably expressing CA-Src were then transiently transfected with the plasmid harboring the eGFP-tagged β_2 AR. The influence of expression of CA-Src on the localization of GFP-tagged β_2 AR was explored (Figure 8). The epifluorescence images reveal that in A431 cells, in the absence of insulin stimulation, the β_2 ARs are localized largely to the cell membrane (Figure 8A). Expression of the CA-Src, in contrast, provoked redistribution of the β_2 ARs to the perinuclear space (Figure 8B). In cells expressing the CA-Src, the pattern of β_2 AR-GFP intracellular localization resembled the one induced by cell stimulation with insulin (Figures 2 and 8). Expression of CA-Src led to the activation of PI3-kinase activity (Figure 8, C and D). Thus, either activation of Src by insulin or the introduction of a constitutively active version of Src leads to sequestration of the β_2 ARs from the cell membrane, a hallmark of the counterregulatory effect of insulin on catecholamine action.

Figure 8. Expression of constitutively active Src activates PI3-kinase and provokes β_2 AR sequestration in the absence of insulin stimulation. A431 clones stably transfected with the constitutively active mutant of Src (CA-Src) or with empty vector (control) were transiently transfected with GFP-tagged β_2 ARs. At 30 h posttransfection, the clones were examined by epifluorescence microscopy (A and B). The images displayed are representative of numerous fields in replicate cultures. The PI3-kinase activity was assayed in the wild-type A431 cells and the clones expressing the constitutively active Src mutant under basal, unstimulated conditions (C and D). The image shown is representative of at least three separate experiments (C), data from which are summarized in D.

DISCUSSION

Src plays a pivotal role of many signaling pathways (Thomas and Brugge, 1997; Martin, 2001). Receptor-based pathways known to couple with Src kinases include integrins, cytokine receptors, immune recognition receptors, various ion channels, and GPCRs (Abram and Courtneidge, 2000).

Figure 9. Schematic model of insulin counterregulation of β_2 ARs. The phosphorylation of Y350 of the β_2 -adrenergic receptor creates an SH2-binding site to which Src is recruited upon insulin stimulation. The β_2 AR is organized in a complex with protein kinase A, protein kinase C, GRK2, and other signaling elements by the AKAP 250 scaffold protein gravin. The effects of insulin can be mimicked by the expression of constitutively activated Src. Inhibition of Src kinase with PP2, by expression of a DN-Src mutant, or the Y350F mutation of the β_2 -adrenergic receptor abolishes the ability of Src to be recruited/activated in response to β -adrenergic agonist and thereby precludes both desensitization and eventually full internalization of the receptor–Src complex. In this manner, the internalization of β_2 AR in response to insulin has many similarities to the internalization stimulated by β -adrenergic agonists after activation. GLUT4 glucose transporters, in contrast to the situation for β_2 ARs, are localized within the cell in the absence

of insulin. Stimulation by insulin leads to a translocation of GLUT4-laden vesicles to the cell membrane for fusion. Thus, GLUT4 and the β_2 -adrenergic receptor seem to traffic in opposite manners in response to insulin. Whether these two signaling molecules can be localized to the same vesicles, or different vesicles, remains to be established.

Src family kinases phosphorylate substrates that regulate cellular events such as transcription, differentiation, adhesion/migration, cell cycle progression, and apoptosis. Src itself, Fyn, Lyn, Lck, Yes, Hck, and other Src family members have been implicated in a variety of these events, depending on the cellular context of the signaling (Thomas and Brugge, 1997). Fyn has been shown to be involved in insulin growth factor-1 signaling events (Sun *et al.*, 1996), but links between Src and insulin signaling are only first revealed in the present work.

Insulin signaling dominates two cellular events, mitogenesis, via the mitogen-activated protein kinase cascade, and metabolic regulation, highlighted by insulin action at skeletal muscle, liver, and adipose tissue (Czech and Corvera, 1999; Olefsky, 1999; Saltiel and Kahn, 2001). Catecholamines generally act in opposition to insulin. Catecholamines stimulate glycogen breakdown, protein degradation, gluconeogenesis, and lipolysis, whereas insulin alone acts to counteract each of these major metabolic pathways. The ability of insulin to counterregulate the β_2 ARs is an essential element of insulin action (Morris and Malbon, 1999). Insulin has been shown to provoke the phosphorylation of β_2 AR on specific tyrosyl residues confined to the cytoplasmic, C-terminal tail of the receptor (Karoer *et al.*, 1995; Doronin *et al.*, 2000). Phosphorylation of Y350 creates a docking site for proteins with SH2 domains (Baltensperger *et al.*, 1996). The integrity of this residue and its phosphorylation in response to insulin are essential for the counterregulation of β_2 AR function and β_2 AR sequestration by insulin (Shih and Malbon, 1998).

Sequestration of β_2 ARs in response to insulin is equal to or greater than that stimulated by β -adrenergic agonist, translocating up to 85% of the cellular complement of β_2 ARs away from the cell membrane (Karoer *et al.*, 1998). Src has been implicated in agonist-induced sequestration of the β_2 AR, through its ability to phosphorylate and activate the G protein-coupled receptor kinase (GRK2) (Ruiz-Gomez and Mayor, 1997; Sarnago *et al.*, 1999; Fan *et al.*, 2001a). In the current study, we demonstrate that counterregulation of β_2 ARs by insulin requires Src activation. We speculate that the mobilization and activation of Src by the β_2 AR signaling complex in response to insulin may lead subsequently to activation of GRK2 (Sarnago *et al.*, 1999), which would also act to terminate β_2 AR signaling in counterregulation by insulin.

Based upon the advanced knowledge of Src structure and biology, we were surprised to find no literature implicating Src signaling in insulin action. Our results clearly demonstrate that Src is activated and associates with β_2 AR signaling complexes in response to insulin. Src, but not Lyn, Fyn, or Yes, displays increased insulin-induced association with β_2 AR signaling complexes. Inhibition of Src blocks insulin-stimulated β_2 AR sequestration. This insulin-induced activation of Src required for β_2 AR sequestration was observed not only in studies performed in human epidermoid carcinoma A431 cells but also in the mouse 3T3-L1 adipocytes, a well-accepted model for study of insulin action (Green and Kehinde, 1975). In A431 cells, inhibition of Src with PP2 blocks the sequestration of β_2 ARs and the well-known translocation of GLUT4 to the cell membrane in response to insulin.

The interrelationships between insulin and catecholamine action are many in metabolic regulation, epitomized by the counterregulation of β -catecholamine action exerted through insulin-stimulated phosphorylation of the β_2 AR at two dominant sites, Y350 and Y364, both located in the cytoplasmic, C-terminal tail of the receptor (Figure 9). These sites of phosphorylation have been demonstrated in vivo through metabolic labeling and peptide chemistry as well as in vitro by using purified insulin receptor and β_2 ARs (Karoor *et al.*, 1995; Baltensperger *et al.*, 1996). Phosphorylation of Y364 generates a form of the phospho- β_2 AR that is a potent, feedback inhibitor of the insulin receptor tyrosine kinase (Doronin *et al.*, 2002). Phosphorylation of the Y350 residue impairs the ability of the β_2 AR to signal to Gs and facilitates the sequestration of the β_2 AR in response to insulin. We found that the Y350F mutation blocks the ability of insulin to counterregulate the β_2 AR- and insulin-induced receptor sequestration (Karoor *et al.*, 1995). Thus, the ability of this site to be phosphorylated and therefore to create a docking site for proteins with SH2 domains is essential for many regulatory events designed to uncouple the β_2 AR and sequester it away from the cell membrane.

Still unresolved is the question how Src is activated in response to insulin. Based upon several lines of data demonstrating Src involvement in agonist-induced sequestration of β_2 ARs and insulin-stimulated sequestration of β_2 ARs, the answer seems very interesting to ponder. We speculate that the β_2 ARs, upon tyrosyl phosphorylation in response to insulin, provide a docking site for Src that may facilitate activation of Src itself (Figure 9). Some Src associated with the β_2 AR signaling complex in the basal state does not seem to be fully activated, perhaps representing Src binding to other elements in the β_2 AR signaling complex (Fan *et al.*, 2001a,b). Displacement of the intramolecular interactions of the SH2 domain by an SH2 docking site like that in the phosphorylated β_2 ARs and subsequent autophosphorylation of Src Y416, however, are both well-known mechanisms that promote activation of Src phosphotyrosine kinases. Mutagenesis studies of the β_2 AR Y350 site support the first tenet, whereas the studies performed with antibodies specific for the phosphorylated Y416 motif demonstrate the second tenet. The activation of Src seems essential for β_2 AR sequestration based upon three findings: the ability of PP2 to inhibit insulin-induced β_2 AR sequestration; the ability of depletion of Src with antisense morpholinos to block insulin-stimulated β_2 AR sequestration; and the ability of the constitutively active version of Src to provoke β_2 AR sequestration in the absence of insulin stimulation.

In A431 cells, PP2 blocked not only the activation of Src but also the activation of PI3-kinase in response to insulin (Figure 9). If Src activity were required for insulin activation of PI3-kinase, one might expect that PP2 would block all PI3-kinase-dependent downstream events. The recent observation that PP2 does not block insulin-stimulated glucose uptake in mouse 3T3-L1 adipocytes would seem to argue against this obligate role of Src (Pessin and Saltiel, 2000; Imamura *et al.*, 2001). It has been shown for at least the GLUT4 translocation response that PI3-kinase activation is not required for insulin action, and the CAP/Cbl pathway might provide an alternative means to translocate GLUT4 in response to insulin (Pessin and Saltiel, 2000). In this regard, it is of interest that for the A431 cells not only does PP2 block insulin-stimulated β_2 AR sequestration

from the plasma membrane but also GLUT4 translocation to the cell membrane in response to insulin. The cellular "context" of insulin signaling obviously plays an important role and the current results demonstrate that Src activation may be important for insulin signaling in cellular events other than β_2 AR sequestration, for example, GLUT4 translocation. Much further work will be required to define the full family of GPCRs whose trafficking is influenced by insulin and/or other growth factors. Clearly, the well-known counterregulatory effects of insulin on catecholamine action include a central role for Src.

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REFERENCES

- Abram, C.L., and Courtneidge, S.A. (2000). Src family tyrosine kinases and growth factor signaling. *Exp. Cell Res.* 254, 1–13.
- Baltensperger, K., Karoor, V., Paul, H., Ruoho, A., Czech, M.P., and Malbon, C.C. (1996). The β -adrenergic receptor is a substrate for the insulin receptor tyrosine kinase. *J. Biol. Chem.* 271, 1061–1064.
- Carman, C.V., and Benovic, J.L. (1998). G-protein-coupled receptors: turn-ons and turn-offs. *Curr. Opin. Neurobiol.* 8, 335–344.
- Chen, S., Teicher, L.C., Kazim, D., Pollack, R.E., and Wise, L.S. (1989). Commitment of mouse fibroblasts to adipocyte differentiation by DNA transfection. *Science* 244, 582–585.
- Cong, M., Perry, S.J., Lin, F.T., Fraser, I.D., Hu, L.A., Chen, W., Pitcher, J.A., Scott, J.D., Lefkowitz, R.J. (2001). Regulation of membrane targeting of the G protein-coupled receptor kinase 2 by protein kinase A and its anchoring protein AKAP79. *J. Biol. Chem.* 276, 15192–15199.
- Czech, M.P., and Corvera, S. (1999). Signaling mechanisms that regulate glucose transport. *J. Biol. Chem.* 274, 1865–1868.
- Delavier-Klutchko, C., Hoebeke, J., and Strosberg, A.D. (1984). The human carcinoma cell line A431 possesses large numbers of functional β -adrenergic receptors. *FEBS Lett.* 169, 151–155.
- Dodge, K., and Scott, J.D. (2000). AKAP79 and the evolution of the AKAP model. *FEBS Lett.* 476, 58–61.
- Doronin, S., Lin, F., Wang, H., and Malbon, C.C. (2000). The full-length, cytoplasmic C-terminus of the β_2 -adrenergic receptor expressed in *E. coli* acts as a substrate for phosphorylation by protein kinase A, insulin receptor tyrosine kinase, GRK2, but not protein kinase C and suppresses desensitization when expressed in vivo. *Protein Expr. Purif.* 20, 451–461.
- Doronin, S., Wang, H.Y., Malbon, C.C. (2002). Insulin stimulates phosphorylation of the β_2 -adrenergic receptor by the insulin receptor, creating a potent feedback inhibitor of its tyrosine kinase. *J. Biol. Chem.* 277, 10698–10703.
- Edwards, A.S., and Scott, J.D. (2000). A-kinase anchoring proteins: protein kinase A and beyond. *Curr. Opin. Cell Biol.* 12, 217–221.
- Fan, G., Shumay, E., Malbon, C.C., and Wang, H. (2001a). c-Src tyrosine kinase binds the β_2 -adrenergic receptor via phospho-Tyr-350, phosphorylates G-protein-linked receptor kinase 2, and mediates agonist-induced receptor desensitization. *J. Biol. Chem.* 276, 13240–13247.
- Fan, G., Shumay, E., Wang, H.Y., Malbon, C.C. (2001b). The scaffold protein gravin (AKA P250) binds the β_2 -adrenergic receptor via the

- receptor cytoplasmic R329 to L413 domain and provides a mobile scaffold during desensitization. *J. Biol. Chem.* 276, 24005–24014.
- Fraser, I.D., Cong, M., Kim, J., Rollins, E.N., Daaka, Y., Lefkowitz, R.J., and Scott, J.D. (2000). Assembly of an A kinase-anchoring protein- β (2)-adrenergic receptor complex facilitates receptor phosphorylation and signaling. *Curr. Biol.* 10, 409–412.
- Gagnon, A.W., Kallal, L., and Benovic, J.L. (1998). Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the β 2-adrenergic receptor. *J. Biol. Chem.* 273, 6976–6981.
- George, S.T., Berrios, M., Hadcock, J.R., Wang, H.Y., and Malbon, C.C. (1988). Receptor density and cAMP accumulation: analysis in CHO cells exhibiting stable expression of a cDNA that encodes the β 2-adrenergic receptor. *Biochem. Biophys. Res. Commun.* 150, 665–672.
- Green, H., and Kehinde, O. (1975). An established preadipose cell line and its differentiation in culture. II. Factors affecting the adipose conversion. *Cell* 5, 19–27.
- Imamura, T., Huang, J., Dalle, S., Ugi, S., Usui, I., Luttrell, L.M., Miller, W.E., Lefkowitz, R.J., and Olefsky, J.M. (2001). β -Arrestin-mediated recruitment of the Src family kinase Yes mediates endothelin-1-stimulated glucose transport. *J. Biol. Chem.* 276, 43663–43667.
- Jho, E.H., Davis, R.J., and Malbon, C.C. (1997). c-Jun amino-terminal kinase is regulated by $\text{G}\alpha_{12}/\text{G}\alpha_{13}$ and obligate for differentiation of P19 embryonal carcinoma cells by retinoic acid. *J. Biol. Chem.* 272, 24468–24474.
- Kallal, L., Gagnon, A.W., Penn, R.B., and Benovic, J.L. (1998). Visualization of agonist-induced sequestration and down-regulation of a green fluorescent protein-tagged β 2-adrenergic receptor. *J. Biol. Chem.* 273, 322–328.
- Karoor, V., Baltensperger, K., Paul, H., Czech, M.P., and Malbon, C.C. (1995). Phosphorylation of tyrosyl residues 350/354 of the β -adrenergic receptor is obligatory for counterregulatory effects of insulin. *J. Biol. Chem.* 270, 25305–25308.
- Karoor, V., and Malbon, C.C. (1998). G-protein-linked receptors as substrates for tyrosine kinases: cross-talk in signaling. *Adv. Pharmacol.* 42, 425–428.
- Karoor, V., Wang, L., Wang, H.Y., and Malbon, C.C. (1998). Insulin stimulates sequestration of β -adrenergic receptors and enhanced association of β -adrenergic receptors with Grb2 via tyrosine 350. *J. Biol. Chem.* 273, 33035–33041.
- Kassis, S., Olasmaa, M., Terenius, L., and Fishman, P.H. (1987). Neuropeptide Y inhibits cardiac adenylate cyclase through a pertussis toxin-sensitive G protein. *J. Biol. Chem.* 262, 3429–3431.
- Lefkowitz, R.J. (1998). G protein-coupled receptors. III. New roles for receptor kinases and β -arrestins in receptor signaling and desensitization. *J. Biol. Chem.* 273, 18677–18680.
- Lin, F., Wang, H., and Malbon, C.C. (2000). Gravin-mediated formation of signaling complexes in β 2-adrenergic receptor desensitization and resensitization. *J. Biol. Chem.* 275, 19025–19034.
- Lohse, M.J., Benovic, J.L., Caron, M.G., and Lefkowitz, R.J. (1990). Multiple pathways of rapid β 2-adrenergic receptor desensitization. Delineation with specific inhibitors. *J. Biol. Chem.* 265, 3202–3211.
- Luttrell, L.M., Hawes, B.E., van Biesen, T., Luttrell, D.K., Lansing, T.J., and Lefkowitz, R.J. (1996). Role of c-Src tyrosine kinase in G protein-coupled receptor- and $\text{G}\beta\gamma$ subunit-mediated activation of mitogen-activated protein kinases. *J. Biol. Chem.* 271, 19443–19450.
- Martin, G.S. (2001). The hunting of the Src. *Nat. Rev. Mol. Cell. Biol.* 2, 467–475.
- Miller, W.E., Maudsley, S., Ahn, S., Khan, K.D., Luttrell, L.M., and Lefkowitz, R.J. (2000). β -Arrestin1 interacts with the catalytic domain of the tyrosine kinase c-SRC. Role of β -arrestin1-dependent targeting of c-SRC in receptor endocytosis. *J. Biol. Chem.* 275, 11312–11319.
- Morris, A.J., and Malbon, C.C. (1999). Physiological regulation of G protein-linked signaling. *Physiol. Rev.* 79, 1373–1430.
- Obergfell, A., Eto, K., Mocsai, A., Buensuceso, C., Moores, S.L., Brugge, J.S., Lowell, C.A., and Shattil, S.J. (2002). Coordinate interactions of Csk, Src, and Syk kinases with $[\alpha]\text{IIb}[\beta]3$ initiate integrin signaling to the cytoskeleton. *J. Cell Biol.* 157, 265–275.
- Olefsky, J.M. (1999). Insulin-stimulated glucose transport minireview series. *J. Biol. Chem.* 274, 1863.
- Pessin, J.E., and Saltiel, A.R. (2000). Signaling pathways in insulin action: molecular targets of insulin resistance. *J. Clin. Invest.* 106, 165–169.
- Ruiz-Gomez, A., and Mayor, F., Jr. (1997). β -Adrenergic receptor kinase (GRK2) colocalizes with β -adrenergic receptors during agonist-induced receptor internalization. *J. Biol. Chem.* 272, 9601–9604.
- Saltiel, A.R., and Kahn, C.R. (2001). Insulin signaling and the regulation of glucose and lipid metabolism. *Nature* 414, 799–806.
- Sarnago, S., Elorza, A., and Mayor, F., Jr. (1999). Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by Src tyrosine kinase. *J. Biol. Chem.* 274, 34411–34416.
- Shenoy, S.K., McDonald, P.H., Kohout, T.A., and Lefkowitz, R.J. (2001). Regulation of receptor fate by ubiquitination of activated β 2-adrenergic receptor and β -arrestin. *Science* 294, 1307–1313.
- Shih, M., Lin, F., Scott, J.D., Wang, H.Y., and Malbon, C.C. (1999). Dynamic complexes of β 2-adrenergic receptors with protein kinases and phosphatases and the role of gravin. *J. Biol. Chem.* 274, 1588–1595.
- Shih, M., and Malbon, C.C. (1994). Oligodeoxynucleotides antisense to mRNA encoding protein kinase A, protein kinase C, and β -adrenergic receptor kinase reveal distinctive cell-type-specific roles in agonist-induced desensitization. *Proc. Natl. Acad. Sci. USA* 91, 12193–12197.
- Shih, M., and Malbon, C.C. (1996). Protein kinase C deficiency blocks recovery from agonist-induced desensitization. *J. Biol. Chem.* 271, 21478–21483.
- Shih, M., and Malbon, C.C. (1998). Serum and insulin induce a Grb2-dependent shift in agonist affinity of β -adrenergic receptors. *Cell. Signaling* 10, 575–582.
- Song, X., Zheng, X., Malbon, C.C., and Wang, H. (2001). $\text{G}\alpha_{12}$ enhances in vivo activation of and insulin signaling to GLUT4. *J. Biol. Chem.* 276, 34651–34658.
- Sun, X.J., Pons, S., Asano, T., Myers, M.G., Jr., Glasheen, E., and White, M.F. (1996). The Fyn tyrosine kinase binds Irs-1 and forms a distinct signaling complex during insulin stimulation. *J. Biol. Chem.* 271, 10583–10587.
- Thomas, S.M., and Brugge, J.S. (1997). Cellular functions regulated by Src family kinases. *Annu. Rev. Cell. Dev. Biol.* 13, 513–609.
- Wang, H., Lipfert, L., Malbon, C.C., and Bahouth, S. (1989a). Site-directed anti-peptide antibodies define the topography of the β -adrenergic receptor. *J. Biol. Chem.* 264, 14424–14431.
- Wang, H.Y., Berrios, M., Hadcock, J.R., and Malbon, C.C. (1991). The biology of β -adrenergic receptors: analysis in human epidermoid carcinoma A431 cells. *Int. J. Biochem.* 23, 7–20.
- Wang, H.Y., Berrios, M., and Malbon, C.C. (1989b). Localization of β -adrenergic receptors in A431 cells in situ. Effect of chronic exposure to agonist. *Biochem. J.* 263, 533–538.
- Wang, H., Doronin, S., and Malbon, C.C. (2000). Insulin activation of mitogen-activated protein kinases Erk1,2 is amplified via β -adrenergic receptor expression and requires the integrity of the Tyr350 of the receptor. *J. Biol. Chem.* 275, 36086–36093.
- Wang, H.Y., Watkins, D.C., and Malbon, C.C. (1992). Antisense oligodeoxynucleotides to GS protein α -subunit sequence accelerate differentiation of fibroblasts to adipocytes. *Nature* 358, 334–337.