Research Article

Sigma-1 Receptors Bind Cholesterol and Remodel Lipid Rafts in Breast Cancer Cell Lines

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Abstract

Lipid rafts are membrane platforms that spatially organize molecules for specific signaling pathways that regulate various cellular functions. Cholesterol is critical for liquid-ordered raft formation by serving as a spacer between the hydrocarbon chains of sphingolipids, and alterations in the cholesterol contents of the plasma membrane causes disruption of rafts. The role that σ receptors play in cancer is not clear, although it is frequently up-regulated in human cancer cells and tissues and σ receptors inhibit proliferation in carcinoma and melanoma cell lines, induce apoptosis in colon and mammary carcinoma cell lines, and reduce cellular adhesion in mammary carcinoma cell lines. In this study, we provide molecular and functional evidence for the involvement of the enigmatic σ1 receptors in lipid raft modeling by σ1 receptor–mediated cholesterol alteration of lipid rafts in breast cancer cell lines. Cholesterol binds to cholesterol recognition domains in the COOH terminus of the σ1 receptor. This binding is blocked by σ receptor drugs because the cholesterol-binding domains form part of the σ receptor drug-binding site, mutations of which abolish cholesterol binding. Furthermore, we outline a hypothetical functional model to explain the myriad of biological processes, including cancer, in which these mysterious receptors are involved. The findings of this study provide a biological basis for the potential therapeutic applications of lipid raft cholesterol regulation in cancer therapy using σ receptor drugs. [Cancer Res 2007; 67(23):11166–75]

Introduction

The plasma membrane of eukaryotic cells form liquid-ordered microdomains enriched with cholesterol and sphingomyelin that spatially organize molecules for specific signaling pathways that regulate a variety of processes (1). σ Receptors were first thought to be a novel type of opiate receptor (2); subsequent studies showed that the σ receptors were able to bind a variety of pharmacologically effective drugs, including benzomorphans, neuroleptics, antidepressants, cocaine, peptides related to neuropeptide Y, and neurosteroids (for review, see ref. 3), although intriguingly no known natural specific ligand(s) have been discovered. The σ receptors have been classified into three subtypes; thus far, the only gene that has been cloned is the *σ1 receptor* subtype (4, 5). The σ1 receptor protein does not share any homology with any known mammalian protein; however, it has some amino acid homology with yeast sterol isomerases (4). The σ1 receptors are transmembrane proteins that are reported to be localized to plasma membranes (6, 7), perinuclear areas, and in regions of cell-to-cell communication (8) and are proposed to possess cytoplasmic NH_2 and COOH termini (6). However, σ1 receptor drugs trigger translocation of the σ1 receptor from the cytosol to the vicinity of the cell membrane, indicating the dynamic nature of this receptor localization (9). Splice variants of the σ1 receptor were found to be nonfunctional in drug-binding assays, suggesting that the drug-binding site is located within the COOH terminus (10). Moreover, site-directed mutagenesis studies have identified two amino acids in the COOH terminus, D126 and E172, that are obligatory for ligand binding (11).

Sigma receptors are implicated pharmacologically in a multitude of functions, disorders, and cellular processes (6, 12, 13). Surprisingly, the σ1 receptor knockout mouse shows no obvious phenotype (14, 15). The role that σ1 receptors play in cancer is not clear (for review, see ref. 16), although it is frequently up-regulated in human cancer cells and tissues (7, 16, 17) and σ receptors inhibit proliferation in carcinoma and melanoma cell lines, induce apoptosis in colon and mammary carcinoma cell lines, and reduce cellular adhesion in mammary carcinoma cell lines (for review, see ref. 16). Moreover, numerous preclinical studies have evaluated the usefulness of radiolabeled σ drugs (particularly σ2 receptor drugs), as tumor-imaging agents (16).

Experiments indicate that G proteins or protein phosphorylation (18, 19) do not mediate σ1 receptor responses and that signal transduction requires close proximity between the receptor and its target molecule (18). Recently, the presence of σ1 receptors in both raft and nonraft fractions was shown (20), and it was postulated that σ1 receptors cause the remodeling of lipid rafts, by increasing the level of lipid raft–associated cholesterol through an unknown mechanism (21). Moreover, Hayashi and Su (22) proposed that σ1 receptors are involved in regulation of galactosylceramide-enriched lipid microdomains and oligodendrocyte differentiation in the nervous system. Hayashi and Su (23) found σ1 receptors in detergent-resistant microdomains, which differed from those of classic, glycosphingolipid-containing lipid rafts in that they possessed a higher buoyant density than classic lipid rafts but retained high cholesterol content. Interestingly, both σ1 receptors and lipid raft levels are elevated in cancer cell lines (7, 24).

Materials and Methods

Cell culture. Human breast cancer cell lines (MDA-MB-231) and human embryonic kidney cell line (HEK) were obtained from the American Type Culture Collection and cultured in DMEM (Life Technologies) containing 10% fetal bovine serum and 4 mmol/L glutamine with penicillin-streptomycin. All cell lines were maintained in a 37°C CO_2 incubator in 100-mm culture dishes. For certain adhesion experiments, cells were plated

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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onto dishes containing extracellular matrix (ECM) proteins (collagen, fibronectin, or laminin). Plates were coated in a solution containing 125 μg/mL of ECM protein in PBS for 1 h at 37°C before washing thrice with PBS and once with DMEM.

Expression and purification of σ1 receptor from HEK cells. The σ1 receptor gene was cloned into pCDNA3.1-c-myc (Invitrogen) so that a c-myc tag was fused to the COOH terminus. The plasmid was transfected into 100-mm culture dishes containing HEK 293 cells using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. As a control, an empty plasmid was also transfected into HEK cells. Four days posttransfection, σ1 receptor tagged with c-myc was purified from these cells using a Profound c-myc tag purification kit (Pierce).

Drugs, buffers, reagents, and antibodies. SKF10047 (Sigma), the prototypical σ1 receptor drug, was used at a concentration of 100 μmol/L, which has been shown to be the optimal concentration when used in whole-cell situations (7). Please note that the σ receptor drug concentrations used for functional assays on whole cells are higher than concentrations for radiolabeled σ receptor agonists used in "receptor binding" studies on isolated membrane preparations. Buffers are listed in Supplementary Material.

The following peptides were obtained from Biopeptide:

$\sigma1_{161\text{-}180(myc)}$ EQKLISEEDLAVEWGPNTWMVEYGRGVIPS,
$\sigma1_{161\text{-}180(myc)Y173S}$ EQKLISEEDLAVEWGPNTWMVESGRGVIPS,
$\sigma1_{191\text{-}210(myc)}$ EQKLISEEDLFSTQDFLTLFYTLRSYARGL,
$\sigma1_{191\text{-}210(myc)Y201/6S}$ EQKLISEEDLFSTQDFLTLFSTLRSSARGL,
$Env_{686\text{-}705(myc)}$ EQKLISEEDLFDITNWLWYIRLFIMIVGGL, and
$Env_{686\text{-}705}$ FDITNWLWYIRLFIMIVGGL.

All peptides were initially dissolved in PBS or PBS with 10% DMSO at 1 mg/mL σ1 receptor antibody as described previously (7). Other antibodies were obtained from commercial suppliers as detailed in Supplementary Material.

Immunostaining of cells for β_1 integrin. Detailed protocol listed is in Supplementary Material. Cells were visualized on a Leica DMIRB confocal with a ×100 objective, and analysis was performed with Leica confocal software.

Protein assays. Samples in a volume of 100 μL were applied to nitrocellulose membrane using a slot blotting apparatus. The blots were blocked with a solution containing 2.5% skimmed milk and 2.5% bovine serum albumin (BSA) in PBST overnight at 4°C before probing with specific antibodies.

Protein samples generally at a concentration of 1 mg/mL were mixed with SDS sample buffer (Sigma) and 5 μg of protein were loaded per lane and separated on acrylamide 4% to 20% gradient Tris-glycine minigels (Cambrex). The proteins were transferred to nitrocellulose membrane for 2 to 4 h at 4°C; transfer was verified using Ponceau red (Sigma) and the blots were blocked with a solution containing 2.5% skimmed milk and 2.5% BSA in TBST overnight at 4°C before probing with specific antibodies. Immunoblots (slot or Western) were probed with antibodies to various epitopes. The antibodies were diluted to 1 μg/mL in PBST with 0.5% skimmed milk and 0.5% BSA and incubated with the blot for 4 h at room temperature. Following four 10-min washes with PBST (with constant agitation) at room temperature, the blots were incubated with anti-mouse or anti-rabbit horseradish peroxidase–conjugated secondary antibodies (at the manufacturers' suggested concentrations) as appropriate, and diluted in PBST with 0.5% skimmed milk and 0.5% BSA for 2 h at room temperature. The blots were again washed as before and then developed with an enhanced chemiluminescence Western blot kit (Amersham). All blots were quantified using NIH IMAGE. Immunoprecipitations were carried out with a kit from Pierce. As controls, the immunoprecipitation was performed in the absence of immunoprecipitation antibody (either σ1 receptor or β_1 integrin) and with beads without protein A attached (to verify nonspecific binding of proteins).

Isolation of lipid raft microdomains, lipid raft staining, and cholesterol assays. About 1×10^8 cells were washed twice in PBS and then treated on ice with 2 mL of lysis buffer containing 1% Triton X-100 in 25 mmol/L Tris-HCl and 140 mmol/L NaCl (pH 8.0). The lysis buffer contained a cocktail of protease inhibitors (Complete EDTA-free; Roche Diagnostics). Cells were left on ice for 20 to 30 min to lyse, and then centrifuged at 10,000 × *g* for 5 min at 4°C to obtain the postnuclear fraction. The quantity of protein in this fraction was calculated using a Bio-Rad protein estimation kit. The postnuclear lysate was adjusted to 50 mg of total protein in 40% (w/v) sucrose and then a 5% to 30% discontinuous sucrose gradient was layered on the top. Typically, 4 mL of 30% sucrose and 3 mL of 5% sucrose were layered on a 3 mL sample in 40% sucrose in a centrifuge tube. Gradients were centrifuged for 16 h at 175,000 × *g* in an SW41 rotor at 4°C. Fractions of 1 mL (typically 10 fractions in total) were collected from the top of the gradient and stored at −80°C. Lipid raft labeling uses a fluorescently labeled cholera toxin subunit B (CT-B), which binds to the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively partitions into lipid rafts. An antibody that specifically recognizes CT-B is then used to cross-link the CT-B–labeled lipid rafts into distinct patches on the plasma membrane, which are visualized by fluorescence microscopy after fixing of the cells with paraformaldehyde. These reagents were provided in a Vybrant Lipid Raft Labeling Kit from Invitrogen. Cholesteryl hemisuccinate was immobilized on diaminodipropylamine agarose (Pierce) with dicyclohexylcarbodiimide (Sigma). At the same time, diaminodipropylamine agarose was reacted without cholesteryl hemisuccinate to serve as a control for nonspecific binding. Beads were blocked in 5% (lipid-free) BSA in PBS for 2 h at room temperature. Beads were stored in PBS with 0.02% NaN_3 at 4°C as 50% bead/PBS slurry. Peptides or proteins were dissolved in 100 μL of PBS with 0.1% Triton X-100 and mixed with 100 μL of cholesteryl beads (or control beads) for 2 h at room temperature. The beads were washed thrice with PBS with 0.05% Triton X-100 using Handee spin cup columns (Pierce), and bound peptides/proteins were eluted by incubating the beads with 100 μL of SDS-PAGE buffer (Sigma) at 80°C for 5 min. Cholesterol was assayed spectrophotometrically using a cholesterol/cholesterol ester quantitation kit (BioVision).

Molecular modeling of σ1 receptor peptides and protein analysis. See Supplementary Material.

σ1 Receptor gene silencing. A σ1 receptor gene silencing vector was constructed using the pSilencer RNAi vector (Ambion) as described previously, which affects both σ1 receptor mRNA and protein levels without effecting basal gene expression levels (7). As a control, a σ1 receptor RNAi randomized sequence was constructed in pSilencer. Transfection was accomplished using Lipofectamine 2000 (Invitrogen) and Opti-MEM (Life Technologies). Transfection efficiency was verified by cotransfecting a green fluorescence protein–expressing plasmid. Silencing efficiency was quantified by protein extraction from cultures 4 days posttransfection and slot blotting 5 μg of total cell protein onto nitrocellulose followed by detection using a σ1 receptor–specific antibody and an antiactinin antibody (as control for nonspecific effects on gene expression; Supplementary Fig. S1). Additionally, mRNA levels were detected for the σ1 receptor using reverse transcription-PCR as described previously (7). Silencing efficiencies of >70% were usually obtained.

Single-cell adhesion measuring assay. This process has been described previously (7).

Biotin surface labeling. Cells (1×10^7) were washed in cold PBS and incubated for 2 h at 4°C with 10 mg/mL sulfo-NHS-LC-biotin (Molecular Probes) in binding buffer, 50 mmol/L $NaHCO_3$ (pH 8.5). The remaining reactive sulfo-NHS-LC-biotin was blocked by adding 2 vol of 100 mmol/L Tris-HCl (pH 8.0) and by further incubation for 1 h. Cells were harvested by incubation twice in cold PBS (pH 7.4) for 2 min and once in PBS, containing 1 mmol/L EDTA, 1% Triton X-100, 150 mmol/L NaCl, and 1 mmol/L DTT, with protease inhibitors. The cells were scraped from the dish, and cell debris was removed by centrifugation at 300 × g for 20 min at 4°C. Biotinylated proteins were purified using Immunopure-immobilized avidin (Pierce). As a control, biotinylated samples were separated by SDS-PAGE, Western blotted, and probed with antibodies to actin to verify nonlabeling of internal proteins.

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Statistical analysis. Data were analyzed by one-way ANOVA. Differences were taken as statistically significant for $P < 0.05$; n represented the number of repeats, whereas N represented the total number of cells tested.

Results

The σ1 receptor contains cholesterol-binding motifs in its COOH terminus. σ1 Receptors are predicted to have at least one (4) or two transmembrane domains (amino acids 9–28 and 81–101; ref. 6) and intracellular NH_2 and COOH termini (6). Transmembrane predictions reveal a further possible region of transmembrane helices (amino acid residues 176–203; Fig. 1*A*), which contain cholesterol-binding domain (CBD) motifs of the sequence L/V-$X_{1\text{-}5}$-Y-$X_{1\text{-}5}$-K/R as described in the benzodiazepine receptor (25) and the HIV envelope protein (26). The sequences VEYGR (single CBD) and LFYTLRSYAR (two overlapping CBDs) from the σ1 receptor are presented schematically in a revised model for the σ1 receptor in Fig. 1*B*. Also shown are amino acid residues known to be obligatory for σ1 receptor drug binding and the splice sites of a non–drug binding splice variant (10, 11).

Do σ1 receptors bind cholesterol? Because σ receptors are reported to be localized to lipid rafts, it is highly likely that they are associated with cholesterol. Therefore, we attempted to produce an assay that is capable of determining true cholesterol binding instead of mere association in a membrane. A c-myc–tagged σ1 receptor (σ1-myc) construct was transfected into the HEK mammalian cell line, and the protein was purified through its myc tag. As expected, bands of ~26 kDa were observed upon

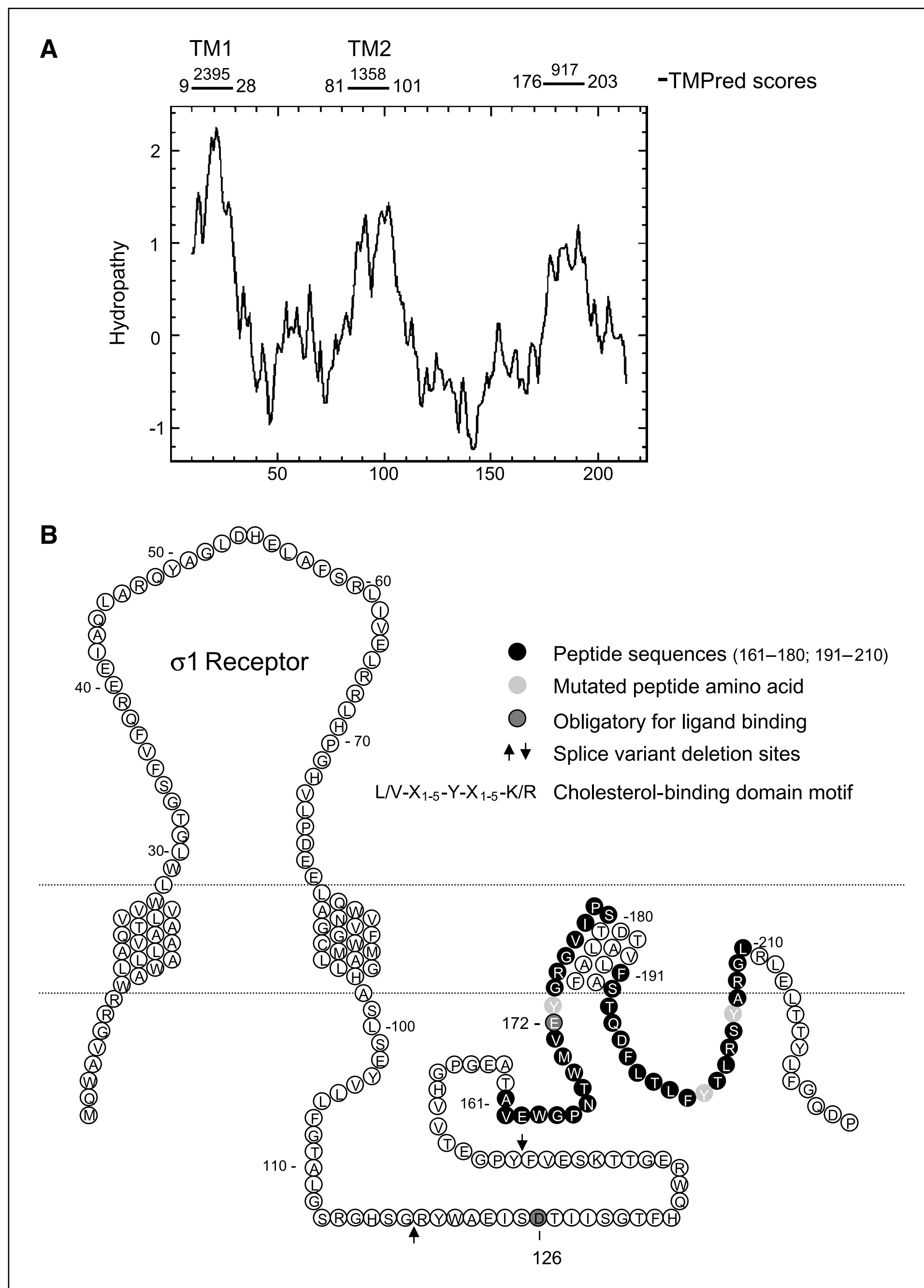

Figure 1. σ1 Receptor contains CBD motifs in its COOH terminus. *A,* transmembrane analysis and hydropathy plots of the σ1 receptor. *B,* σ1 receptor amino acid sequence contains several CBD motifs of the structure L/V-$X_{1\text{-}5}$-Y-$X_{1\text{-}5}$-K/R. The sequences VEYGR (single CBD) and LFYTLRSYAR (two overlapping CBDs) from the σ1 receptor are represented schematically in a model for the σ1 receptor.

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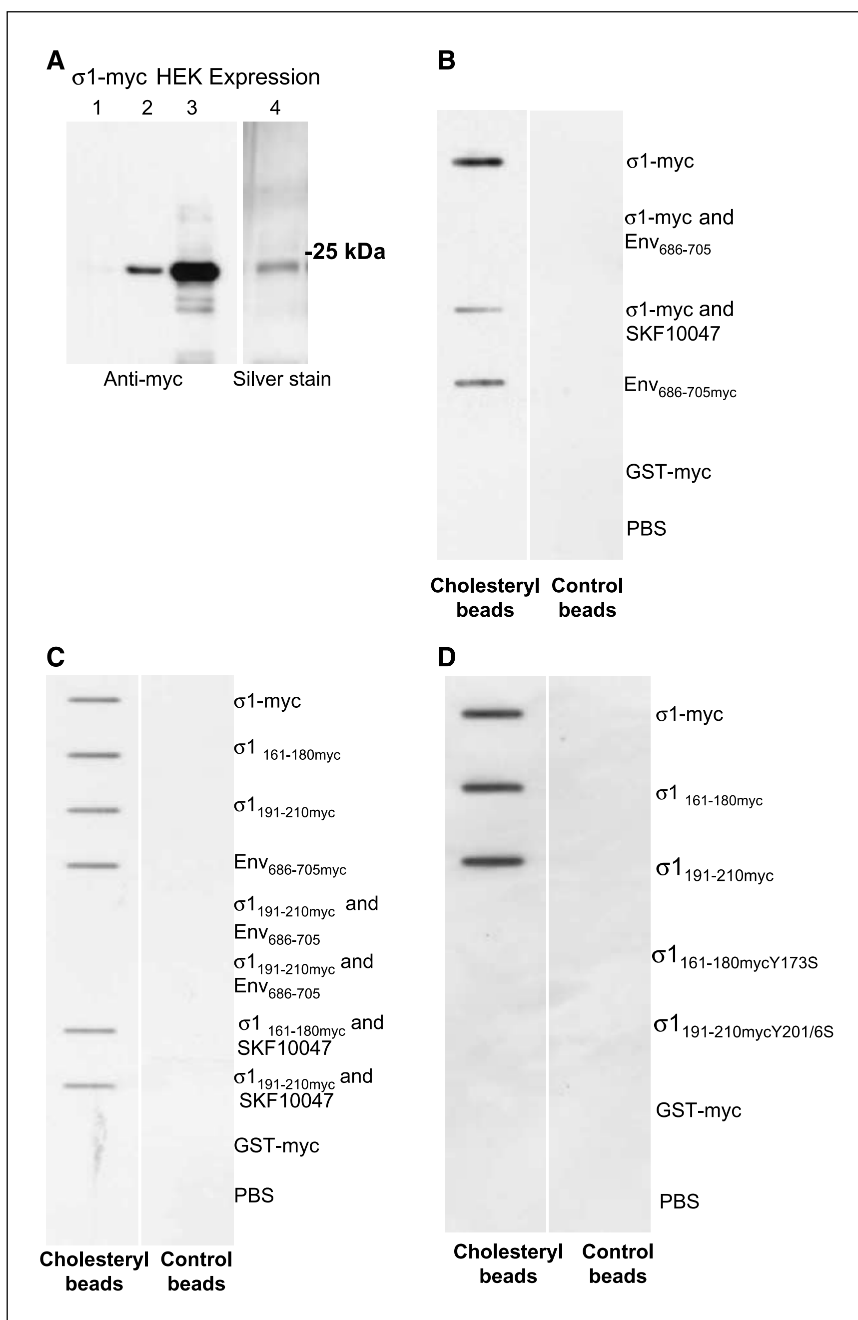

Figure 2. σ1 Receptor binds cholesterol. *A,* expression and purification of the σ1 receptor with a c-myc tag (σ1-myc) in HEK cells. Proteins were characterized by Western blotting (*antibody indicated under blot*) and silver staining of SDS-PAGE gels. *Lane 1,* total cell extract from control-transfected cells; *lane 2,* total cell extract from tagged σ1 receptor–transfected cells; *lanes 3* and *4,* purified σ1 receptor protein. *B,* binding of purified σ1-myc to cholesteryl immobilized to beads. Proteins/peptides eluted from cholesteryl immobilized beads were slot-blotted to nitrocellulose and probed with antibodies to the c-myc tag. Binding of σ1-myc to cholesteryl beads was blocked by a cholesterol-binding peptide from HIV-1 envelope protein ($Env_{686–705}$) and inhibited SKF10047. *C,* binding of σ1 receptor peptides $\sigma1_{161\text{-}180myc}$ and $\sigma1_{191\text{-}210myc}$ to cholesteryl beads and inhibition by cholesterol-binding peptide ($Env_{686–705}$) and partial inhibition by SKF10047. *D,* binding of mutated σ1 receptor peptides $\sigma1_{161\text{-}180myc(Y173S)}$ and $\sigma1_{191\text{-}210myc(Y201/6S)}$ to cholesteryl beads.

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SDS-PAGE and Western blotting (Fig. 2*A*). Cholesterol was immobilized to beads (27) and used to study σ1 receptor binding to cholesterol as described previously for the HIV envelope protein, which also contains a CBD (26). σ1-myc pure protein at a concentration of 100 ng/mL was found to bind to cholesterol beads but not to control beads (Fig. 2*B*). As a positive control, a known cholesterol-binding peptide $Env_{686\text{-}705myc}$ (26) with a myc tag attached was also tested (9 ng/mL). This cholesterol-binding peptide $Env_{686\text{-}705}$ (without a myc tag) at a 100-fold molar excess was found to block binding of σ1-myc protein to cholesterol, indicating that the σ1 receptor binds specifically to cholesterol (Fig. 2*B*). As a negative control, a glutathione *S*-transferase protein with a myc tag was also tested. The σ1 receptor drug SKF10047 (at a 100 μmol/L concentration, ~25-fold molar excess) was found to block the binding of σ1-myc to cholesterol beads (Fig. 2*B*) by 80% (F = 270, $P = 3.2 \times 10^{-6}$, n = 5), demonstrating that the cholesterol-binding sites of the σ1 receptor form part of the drug-binding site for this receptor. Peptides spanning the σ1 receptor CBD motifs were synthesized (Fig. 1*A*) with myc tags attached ($\sigma1_{161\text{-}180myc}$ and $\sigma1_{191\text{-}210myc}$). These peptides at a concentration of 13.5 ng/mL were found to bind to cholesterol beads (Fig. 2*C*). The binding of these peptides to cholesterol beads was blocked by the cholesterol-binding peptide $Env_{686\text{-}705}$ (used at a 100-fold molar excess; Fig. 2*C*). SKF10047 (100 μmol/L) reduced the binding of these peptides to cholesterol beads by a small amount, 39% (F = 58, $P = 1.6 \times 10^{-3}$, n = 5) and 33% (F = 43, $P = 2.8 \times 10^{-3}$, n = 5), respectively (Fig. 2*C*). Analysis of the CBD from the benzodiazepine receptor revealed that mutation of the

tyrosine in the sequence L/V-X_{1-5}-Y-X_{1-5}-K/R abolished binding to cholesterol (25). σ1 Receptor peptides $σ1_{161\text{-}180myc}$ and $σ1_{191\text{-}210myc}$ were synthesized with Y173S and double Y201/6S mutations, respectively. The binding of these mutated peptides to cholesterol beads was abolished (n = 3; Fig. 2*D*), indicating that the cholesterol-binding site lies close to these amino acid residues.

Molecular modeling of σ1 receptor cholesterol-binding peptides and predicted cholesterol docking. To understand the ability of the σ1 receptor to bind cholesterol, molecular modeling studies were undertaken on the cholesterol-binding peptides $σ1_{161\text{-}180}$ and $σ1_{191\text{-}210}$. To determine the cholesterol-σ1 receptor interaction, the following aspects must be considered: (*a*) cholesterol is a planar molecule, thus offering a limited number of conformations; (*b*) its length corresponds to about three helix turns; and (*c*) its small polar head, a hydroxyl group, should be located at the boundary region of the membrane interface (i.e., close to R175 and R208 in our model; ref. 25). All these features simplify the search for docking sites for cholesterol. Almost all the bulky side chains (F, L, or V) are gathered on one helix side of both peptides, which can thus be ruled out for steric hindrance reasons as a part of a cholesterol-binding site. The results show that both peptides may form a pocket where cholesterol could dock (Fig. 3*A* and *B*). This pocket is absent in the mutated peptides (data shown only for $σ1_{191\text{-}210\ Y173S}$), predicting the lack of interaction of these sequences with cholesterol (Fig. 3*A*). Computational docking simulations indicated that, indeed, cholesterol could dock in the pocket of the target most likely in the vicinity of Y173 and Y206 (which is in agreement with the mutational studies) with the polar group of cholesterol rationally buried in the lipid membrane. Docking of cholesterol was not possible in the vicinity of peptides with Y173S or Y201/6S mutations (data shown only for $σ1_{191\text{-}210\ Y173S}$). These results are in concordance to the results obtained for the docking of cholesterol to the CBD of the benzodiazepine receptor (25). Docking of cholesterol within the vicinity of Y201 in $σ1_{191\text{-}210}$ was possible but resulted in the hydroxyl group being exposed to an aqueous environment (data not shown).

Physiologic implications of the σ1 receptors ability to bind cholesterol. The σ receptors have been implicated in a multitude of cellular functions (3, 13, 16). It has been shown that either σ1 receptor drugs or σ1 gene silencing reduce cellular adhesion in mammary carcinoma cell lines (7). In this study, we chose to study the mechanism through which σ1 receptors can affect the adhesion of cancer cells in light of our experiments, demonstrating the ability of the σ1 receptor to bind cholesterol. As previously reported, SKF10047 significantly reduces the adhesion of MDA-MB-231 cells (7) and $β_1$ integrin plays a significant role in the adhesion of MDA-MB-231 mammary cancer cell line (28). Application of SKF10047 or a $β_1$ integrin blocking antibody for 2 h significantly reduced the adhesion by 72% (F = 49, $P = 1.4 \times 10^{-11}$, N = 140, n = 3) and 88% (F = 84, P = 0, N = 140, n = 3; Fig. 4*A*). Dual application of SKF10047 and $β_1$ integrin blocking antibody did not significantly alter the adhesion compared with $β_1$ integrin blocking antibody alone (F = 0.01, P = 0.94, N = 140, n = 3). As previously reported, σ1 receptor silencing significantly reduced the adhesion of MDA-MB-231 cells (7). In this study, we observed a 49% reduction (F = 13, $P = 3.8 \times 10^{-4}$, N = 109, n = 3) in adhesion; however, as for the SKF10047 experiments above, dual σ1 receptor silencing and $β_1$ integrin blocking did not significantly alter the adhesion compared with $β_1$ integrin blocking alone (F = 0.9, P = 0.34, N = 110, n = 3; Fig. 4*A*). Both of these results indicate that $β_1$ integrin blocking and σ1 receptor drugs or σ1 receptor gene silencing have a similar effect. Because dual treatments of $β_1$ integrin blocking and σ1 receptor modulation (with SKF10047 or gene silencing) was not significantly different from $β_1$ integrin blocking alone, we propose that σ1 receptors may be altering cellular adhesion in MDA-MB-231 cells by effecting $β_1$ integrin.

$β_1$ integrin heterodimers are receptors for collagen, fibronectin, and laminin. Therefore, we also tested the effect of SKF10047

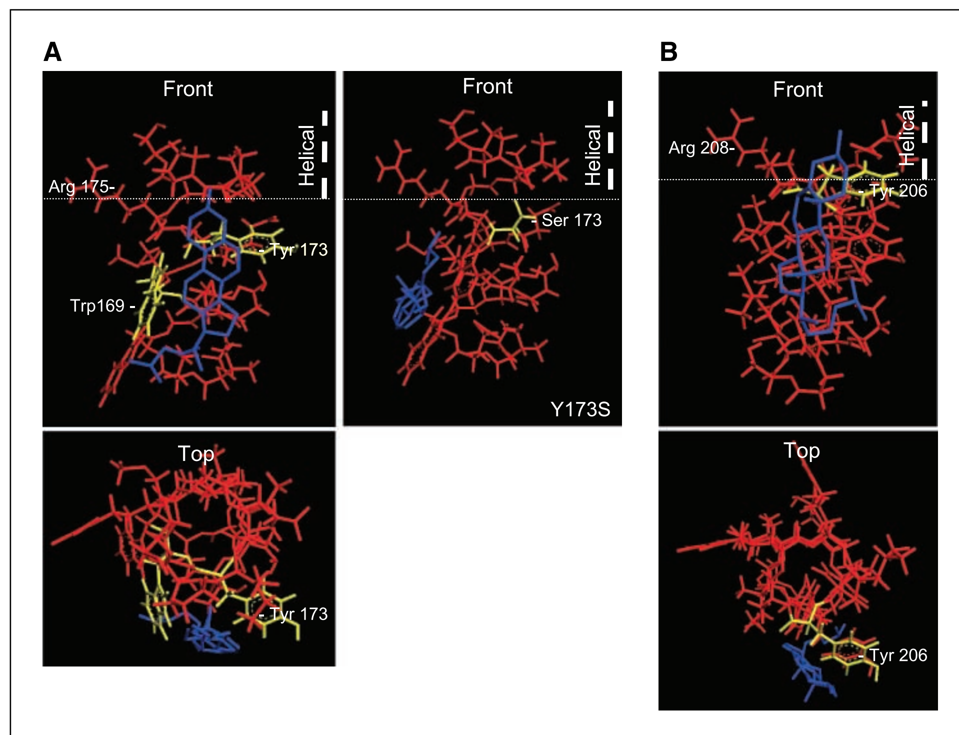

Figure 3. Molecular modeling of σ1 receptor cholesterol-binding peptides and predicted cholesterol docking. σ1 Receptor peptide $σ1_{161\text{-}180}$ (*A*) and $σ1_{191\text{-}210}$ (*B*) were modeled using ArgusLab software ($\phi = -135$ deg, $\psi = -45$ deg, and $\omega = 180$ deg). Following energy minimalization by Universal Force Field prediction, the structures were presented in two different views (*front* and *top*). *Dashed line,* the predicted positions of these peptides in a bilipid layer. The docked location of the cholesterol ($C_{29}H_{50}O$) ligand (*blue molecule*) to each peptide was predicted by using simulated annealing as implemented using ArgusDock. In the mutated peptides $σ1_{161\text{-}180(Y173S)}$ and $σ1_{191\text{-}210(Y201/6S)}$, the cholesterol was docked in a position such that the polar end group was presented to an unfavorable environment.

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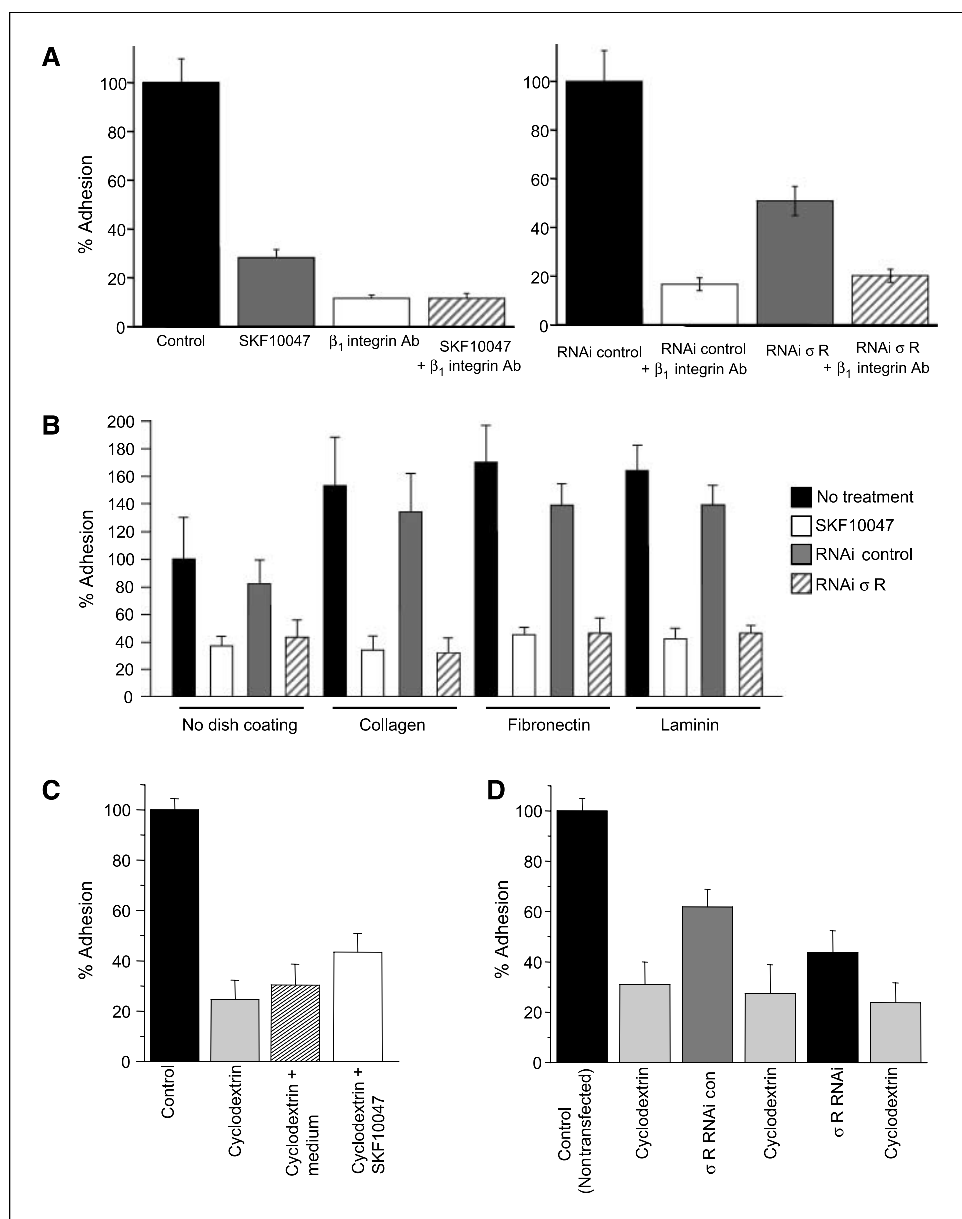

Figure 4. Effect of σ receptors on β_1 integrin–mediated cell adhesion. *A,* MDA-MB-231 cells were either treated with SKF10047, a β_1 integrin blocking antibody (*Ab*), or both for 2 h; the adhesion was measured using single-cell adhesion measuring assay compared with untreated control cells. In a separate experiment, the σ1 receptor in MDA-MB-231 cells was silenced by RNAi or transfected with a control RNA construct. Again, the cells were treated with either a β_1 integrin blocking antibody or medium for 2 h, and the adhesion was measured using single-cell adhesion measuring assay. *B,* MDA-MB-231 cells were plated onto collagen, fibronectin, or laminin-coated plates; cells were either treated with SKF10047 for 2 h or the σ1 receptor was silenced by RNAi or transfected with a control RNA construct; and the adhesion was measured using single-cell adhesion measuring assay. *C,* MDA-MB-231 cells were treated with 2% cyclodextrin for 30 min followed by a 2-h treatment with SKF10047 or medium as control, and the adhesion was measured using single-cell adhesion measuring assay. *D,* the σ1 receptor in MDA-MB-231 cells was silenced by RNAi or transfected with a control RNA construct, and the adhesion was measured by using single-cell adhesion measuring assay after 30-min incubation with 2% cyclodextrin. *Columns,* mean; *bars,* SE.

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treatment and σ1 receptor RNAi on the adhesion of MDA-MB-231 cells to these substrates. As expected, the adhesion of MDA-MB-231 cells seeded onto dishes coated with collagen, fibronectin, or laminin increased compared with cells plated onto uncoated dishes (Fig. 4*B*). Treatment of cells with SKF10047 produced a greater reduction in adhesion (78%, N = 50, n = 3) for cells seeded onto plates coated with collagen compared with cells seeded onto uncoated dishes (63%, N = 50, n = 3; Fig. 4*B*). Also, the reduction in adhesion of MDA-MB-231 cells upon σ1 receptor RNAi (76%, N = 50, n = 3) on dishes coated with collagen was greater than on cells seeded into uncoated dishes (48%, N = 50, n = 3; Fig. 4*B*). Similar results were obtained with dishes coated with fibronectin and laminin (Fig. 4*B*). These results strengthen our hypothesis that σ1 receptors may be altering cellular adhesion in MDA-MB-231 cells by effecting β_1 integrin.

If lipid raft composition is altered by using drugs that affect lipid raft formation such as methyl-β-cyclodextrin (2% for 30 min), which extracts cholesterol from the membrane, then adhesion is dramatically reduced (Fig. 4*C*). Subsequent treatment with SKF10047 did not reduce the adhesion compared with untreated cells (N = 50, n = 3; Fig. 4*C*). Similarly for cyclodextrin-treated cells, σ1 receptor RNAi did not reduce the adhesion of MDA-MB-231 cells compared with control RNAi (N = 50, n = 3; Fig. 4*D*). These data suggest that if lipid rafts composition is disrupted, σ1 receptors are unable to affect cellular adhesion. It seems plausible that σ1 receptor antagonism or interference inhibit breast cancer cell adhesion by displacing σ1 receptors and β_1 integrin outside lipid rafts, the same as methyl-β-cyclodextrin.

Effect of and interaction of σ1 receptors with β_1 integrin. The effect of σ1 receptors on β_1 integrin was investigated. In SKF10047-treated cells or σ1 receptor–silenced cells, no significant difference in surface β_1 integrin staining was observed compared with untreated or control cells (Fig. 5*A*; F = 0.3, P = 0.3, N = 300, n = 3). SKF10047 treatment did not have any significant effect on total β_1

integrin levels (adjusted to loading controls-actin; $F = 0.45$, $P = 0.99$, $n = 5$) or surface expressed (surface biotinylated) β_1 integrin (Fig. 5*B*; $F = 0.02$, $P = 0.99$, $n = 5$), indicating that neither application of SKF10047 nor σ1 receptor silencing changed the surface expression of β_1 integrin. Immunoprecipitations were performed and the σ1 receptor was found to coimmunoprecipitate with β_1 integrin and visa versa (Fig. 5*C*). Furthermore, although the presence of SKF10047 did not effect total σ1 receptor levels, the levels of σ1 receptor that coimmunoprecipitated with β_1 integrin were significantly reduced by 87% upon application of SKF10047 (Fig. 5*D*; $F = 422$, $P = 3.3 \times 10^{-5}$, $n = 5$). Similarly, the levels of β_1 integrin that coimmunoprecipitated with the σ1 receptor were significantly reduced upon application of SKF10047 by 54% (Supplementary Fig. S2; $F = 602$, $P = 1.6$, $n = 3$).

Effect of σ1 receptors on lipid raft membranes. MDA-MB-231 cells were either treated with SKF10047 or the σ1 receptor was silenced using RNAi. Subsequently, the cells were harvested and lipid raft fractions were prepared. Each fraction was slot blotted to nitrocellulose and probed with antibodies to β_1 integrin (which is found in raft and nonraft fractions; ref. 29), flottillin (a lipid raft resident protein), or Na^+/K^+-ATPase (a nonraft protein; Fig. 6*A*). All antibodies were previously tested in SDS-PAGE and Western blots to ensure the specificity of antibody binding and the absence of nonspecific bands. Results are shown in Fig. 6*B*; a reduction of the levels of β_1 integrin in the lipid raft fractions (fractions 4 and 5) was noted upon SKF10047 application by 43% ($F = 120$, $P = 3.9 \times 10^{-4}$, $n = 3$) or σ1 receptor silencing by 41% ($F = 49$, $P = 2.2 \times 10^{-3}$, $n = 3$), showing that in the presence of SKF10047 or σ1 receptor silencing, β_1 integrin moves from lipid raft fractions to nonraft fractions. Additionally, the SKF10047-treated and untreated cells were slot blotted and probed with a σ1 receptor antibody (Fig. 6*A*) and the results were quantified. In untreated MDA-MB-231 cells,

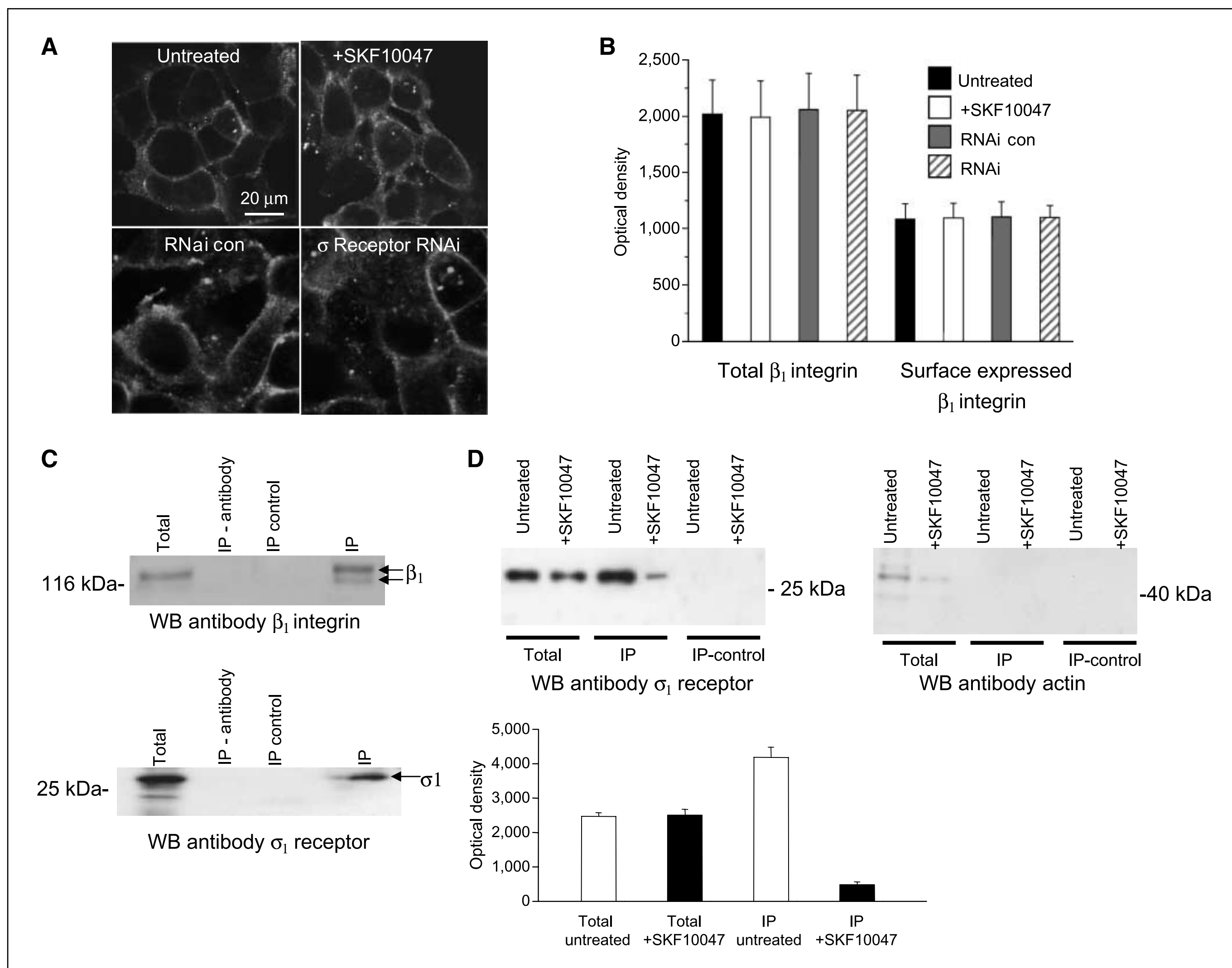

Figure 5. Effect of and interaction of σ1 receptors with β_1 integrin. *A,* effect of SKF10047 and σ1 receptor silencing on surface localized β_1 integrin in MDA-MB-231 cells visualized by immunostaining and confocal imaging. *B,* effect of SKF10047 and σ1 receptor silencing on total and surface localized β_1 integrin as determined by cell surface biotinylation, SDS-PAGE electrophoresis, and Western blotting. *C,* immunoprecipitation (*IP*) between the σ1 receptor and β_1 integrin and vice versa. *Lane 1,* total cell extract. *Lane 2,* immunoprecipitation without antibody. *Lane 3,* immunoprecipitation with control beads (no protein A). *Lane 4,* immunoprecipitation reaction. Western blotting antibody is shown below the blot. *Arrows,* immunoprecipitated protein. *D,* effect of SKF10047 treatment on coimmunoprecipitation of σ1 receptor by β_1 integrin. *Lanes 1* and *2,* total cell extracts. *Lanes 3* and *4,* immunoprecipitation for control and SKF10047-treated cells, respectively. *Lanes 5* and *6,* control bead reactions. Western blot antibodies are shown below each blot. These results were quantified after adjustment for actin loading controls. *Columns,* mean; *bars,* SE.

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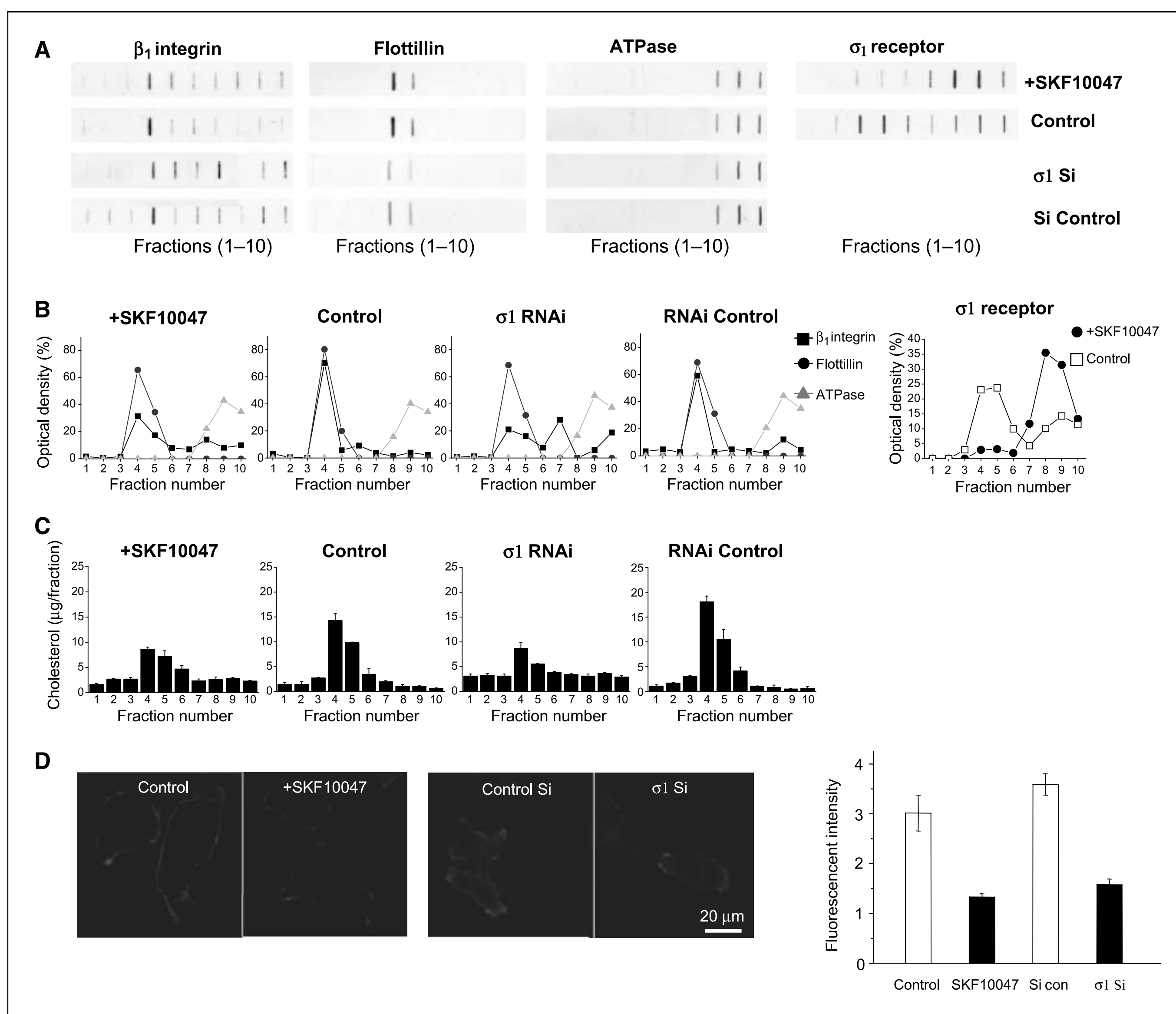

Figure 6. Remodeling of lipid rafts by σ1 receptors. *A,* localization of integrin in lipid raft subcellular fractions after treatment with SKF10047 or σ1 receptor knockdown. Fractions (*1–10*) were slot blotted to nitrocellulose and subsequently probed with antibodies to β_1 integrin, flottilin (lipid raft resident protein), or Na^+/K^+-ATPase (nonlipid raft membrane fraction). SKF10047-treated cells were also blotted with antibodies to the σ1 receptor. *B,* densitometric analysis of above slot-blotted lipid raft subcellular fractions. *C,* cholesterol quantification in lipid raft subcellular fractions. *Columns,* mean; *bars,* SE. *D,* lipid raft staining of cells after SKF10047 treatment and σ1 receptor knockdown (*left*). Quantification of lipid raft staining (*right*). *Columns,* mean; *bars,* SE.

46% of the σ1 receptor protein is present in the lipid raft fractions. Upon SKF10047 treatment, a 7-fold reduction (F = 62, P = 2.1 × 10^{-3}, n = 3) in the amount of σ1 receptor protein present in the lipid raft fractions was observed (Fig. 6*B*). Note that application of 2% methyl-β-cyclodextrin for 30 min to cells reduced the amount of σ1 receptor present in the lipid raft fractions by 7-fold (Supplementary Fig. S3). Cholesterol levels were also quantified from each fraction (Fig. 6*C*), and a reduction in the levels of lipid raft cholesterol was noted upon SKF10047 application by 34% (F = 18, P = 1.3 × 10^{-3}, n = 3) or σ1 receptor silencing by 50% (F = 12, P = 1.6 × 10^{-3}, n = 3). Total cellular cholesterol and total cholesterol in all fractions was not significantly affected (data not shown). We also used lipid raft staining to study the effect of SKF10047 or σ1 receptor silencing on lipid rafts. This labeling uses a fluorescently labeled probe that binds to the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively partitions into lipid rafts. An antibody that recognizes the probe is then used to cross-link the labeled lipid rafts into patches on the plasma membrane. We observed a significant reduction in lipid raft staining (Fig. 6*D*) upon SKF10047 treatment by 56% (F = 26, P = 1.4 × 10^{-6}, N = 500, n = 3) or σ1 receptor silencing by 56% (F = 70, P = 4.8 × 10^{-13}, N = 500, n = 3).

Discussion

We have shown that the σ1 receptor is capable of binding to cholesterol through two CBD motifs [similar to the CBDs found in the peripheral benzodiazepine receptor (25) and the HIV Env protein (26)]. Cholesterol docked to these CBDs as revealed by molecular modeling in a position such that the polar hydroxyl group

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is buried in the membrane with the steroid rings bound to the σ1 receptor at the submembrane interface in a cleft/concavity. It is likely that these CBDs in the COOH terminus of the σ receptor form part of the SKF10047 binding site because binding of the entire σ1 receptor protein to cholesterol was inhibited by SKF10047 and because one of the amino acids that seem to be essential for cholesterol binding (Y173) is adjacent to one of the amino acid residues (D172) found to be obligatory for ligand binding (11). However, other amino acids (e.g., D126) have been found to be obligatory for ligand binding (11) and SKF10047 only minimally inhibited binding of the σ receptor CBD peptides to cholesterol; thus, we deduced that the CBDs partially form the binding site for SKF10047 along with other amino acids (e.g., D126). Interestingly, a study to investigate the interaction of the phenyl ring of cocaine with the σ1 receptor has identified D188 to be close to or in the binding site (30). Our model for the σ1 receptor is consistent with this recent study, suggesting that this third hydrophobic region in the σ1 receptor is close to the σ1 receptor ligand binding site. There is evidence that certain steroid-like substances, such as progesterone, pregnenolone, and DHEA, can bind to σ receptors (31) and it is plausible that these neurosteroids can affect the ability of σ receptors to bind to cholesterol and hence affect lipid rafts. Our experiments also explains why many drugs that bind to the σ1 receptor such as haloperidol and ifenprodil also bind to the product of the yeast gene *ERG2*, which encodes a sterol C8-C7 isomerase (32). The existence of a lipophilic cholesterol-binding site in the σ1 receptor explains the ability of the σ1 receptor protein to bind so many drugs from distinct pharmacologic classes because we postulate that σ drugs like sterol isomerase inhibitors mimic the unstable carbocationic isomerization intermediate (33, 34).

In this study, we chose to study how σ1 receptors may affect the adhesion of cancer cells. σ1 Receptor knockdown resulted in a lowering of the levels of cholesterol in lipid rafts. Additionally, SKF10047 "block" of the σ1 receptor reduced levels of σ1 receptor localized to lipid rafts, indicating that the σ1 receptor can contribute to the cholesterol content of the lipid layers surrounding them and probably any associated molecules, for example, integrins (in this study) or ion channels (6). Interestingly, Kv1.4 and Kv1.5, two of the channels shown (6) to be a target of σ1 receptors, are also associated with cholesterol-rich lipid rafts, and cholesterol depletion dramatically alters the kinetic properties of Kv1.4 and Kv1.5 (35, 36). Many of the pharmacologic effects of σ receptor drugs observed can be explained in terms of cholesterol alteration of lipid rafts, for example, ion channel modulation (19), transporter modulation (37), *N*-methyl-D-aspartate receptor modulation (38), β_1 integrin modulation (this article), and epidermal growth factor modulation (21), because disruption of cholesterol in lipid rafts has been shown to affect the signaling molecules that are present in these rafts (39).

We have shown that σ1 receptors are in a complex with β_1 integrin in breast cancer cell line MDA-MB-231 and previously with Kv1.4 channels in neurophysial terminals (6). We postulate that σ1 receptors can form complexes with signaling molecules and that the action of the σ1 receptor may be to form or to stabilize lipid rafts in the vicinity of these molecules by insertion of cholesterol. Moreover, levels of the σ1 receptor-β_1 integrin complex in lipid raft fractions were lowered upon SKF10047 application. The observed effects of the σ1 receptor drug SKF10047 on cancer cell adhesion and Kv1.4 (6) can be interpreted only if we consider that this drug acts to block the σ1 receptor. Indeed, either application of SKF10047 or transfection of σ1 receptor RNAi had similar effects in that they lowered the level of β_1 integrin in the raft fractions, which thus affected cellular adhesion. On the basis of adhesion, results, and distribution of receptors in the raft and nonraft sections, it should be stressed that breast cancer cell adhesion inhibition require delocalization of σ1 receptors and β_1 integrin outside lipid rafts to occur.

We can hypothesize that the physiologic function of σ1 receptors may be to bind and insert cholesterol into lipid membranes. This is in agreement with the results of Hayashi and Su (23) who also found σ1 receptors in detergent-resistant microdomains but which differed from those of classic, glycosphingolipid-containing lipid rafts in that they possessed a higher buoyant density than classic lipid rafts but retained high cholesterol content. Future experiments will thus focus on proving that the σ1 receptor is capable of directly inserting cholesterol into lipid membranes. It is interesting to note that both σ1 receptor and lipid raft levels are elevated in cancer cells (7, 24). It is postulated that elevated cholesterol levels lead to lipid raft coalescence, which might serve to sequester and thus stimulate "on" signals to an oncogenic pathway (40). Notably, cholesterol depletion of the plasma membrane results in a reduction in raft levels and leads to anoikis-like apoptosis, Bcl-xL down-regulation, caspase-3 activation, and Akt inactivation of various cancer cell types (24). This is remarkably similar to some of the effects reported for σ receptor drugs (16). It is very interesting to find elevated levels of membrane cholesterol in prostate cancer cell lines and breast cancer cell lines compared with their normal counterparts, especially because σ1 receptors and lipid raft levels are elevated in cancer cells (7, 24) and SKF10047 treatment results in a decrease in lipid raft cholesterol. Additionally, elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents (24); we therefore suggest that σ receptor drugs may be used as a therapeutic treatment to lower lipid raft cholesterol in cancer cells, thus inhibiting "on" signals to an oncogenic pathway (40) and resulting in sensitivity to apoptotic drugs.

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