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Plitidepsin Cellular Binding and Rac1/JNK Pathway Activation Depend on Membrane Cholesterol Content[S]

Yajaira Suárez, Laura González-Santiago, Natasha Zarich, Alberto Dávalos, Juan F. Aranda, Miguel A. Alonso, Miguel A. Lasunción, José María Rojas, and Alberto Muñoz

Instituto de Investigaciones Biomédicas "Alberto Sols," Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Madrid, Spain (Y.S., L.G.-S., A.M.); Pharma Mar S.A., Madrid, Spain (Y.S., L.G.-S.); Centro Nacional de Microbiología, Instituto de Salud Carlos III, Madrid, Spain (N.Z., J.M.R.); Hospital Ramón y Cajal, Madrid, Spain (A.D, M.A.L.); Universidad de Alcalá, Madrid, Spain (M.A.L.); and Centro de Biología Molecular "Severo Ochoa," Consejo Superior de Investigaciones Científicas-Universidad Autónoma de Madrid, Madrid, Spain (J.F.A., M.A.A.)

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ABSTRACT

Plitidepsin (aplidin) is a marine cyclic depsipeptide in phase II clinical development against several neoplasias. Plitidepsin is a potent inducer of apoptosis through the sustained activation of Jun N-terminal kinase (JNK). We have reported that this activation depends on the early induction of oxidative stress, activation of Rac1 small GTPase, and the later down-regulation of MKP-1 phosphatase. Using Scatchard and saturation binding analyses, we have found that ^{14}C-labeled plitidepsin binds to a moderately high-affinity receptor (K_d of 44.8 ± 3.1 and 35.5 ± 4.8 nM, respectively) in MDA-MB-231 breast cancer cells. Two minutes after addition to cells, half of the drug was membrane-bound and was subsequently found in the cytosolic fraction. At 4°C, plitidepsin cellular binding was around 10-fold lower than at 37°C but sufficed to induce cell death, suggesting that this process is triggered from the membrane. Depletion of plasma membrane cholesterol by short treatment with methyl-β-cyclodextrin diminished plitidepsin binding and Rac1 and JNK activation. Rac1 is targeted to the plasma membrane by plitidepsin as shown by subcellular fractioning and immunofluorescence analysis followed by confocal microscopy. Methyl-β-cyclodextrin blocked this effect. A subline of HeLa cells (HeLa-R), partially resistant to plitidepsin, showed similar affinity (K_d of 79.5 ± 2.5 versus 37.7 ± 8.2 nM) but 7.5-fold lower binding capacity than wild-type HeLa cells. Moreover, HeLa-R cells had lower total (71%) and membrane (67%) cholesterol content and membrane-bound Rac1, and showed no Rac1 activation upon plitidepsin treatment. In conclusion, cellular plitidepsin uptake and induction of apoptosis via activation of the Rac1-JNK pathway is membrane-cholesterol dependent.

Plitidepsin is a cyclic depsipeptide presently in phase II clinical trials against several neoplasias. It is an extremely potent inducer of apoptosis by caspase-dependent and -independent mechanisms. In human solid tumor cells, this induction depends on the strong, sustained activation of Jun N-terminal kinase (JNK) (Cuadrado et al., 2004). Confirming the crucial role of JNK for plitidepsin action, Losada et al. (2004) have reported that a subline of HeLa cells (HeLa-R) that is partially resistant to the drug shows only slight, transient JNK activation. It is remarkable that HeLa-R cells showed no cross-resistance with several commonly used antitumoral drugs, and their resistance was unrelated to P-glycoprotein expression (Losada et al., 2004). In addition, plitidepsin activates other kinases such as the epidermal growth factor receptor (EGFR), Src, p38 mitogen activated protein kinase, and, in some cell types, extracellular signal-regulated kinase and protein kinase C-δ, which contribute to its cytotoxicity (García-Fernández et al., 2002; Cuadrado et

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ABBREVIATIONS: Chol, cholesterol; GTPase, guanosine-triphosphatase; JNK, Jun N-terminal kinase; EGFR, epidermal growth factor receptor; VEGF, vascular endothelial growth factor; CD, methyl-β-cyclodextrin; HeLa-R, plitidepsin-resistant HeLa cells; wt, wild type; PBS, phosphate-buffered saline; APL, plitidepsin (Aplidin); TCA, trichloroacetic acid; PBS, phosphate-buffered saline; ROS, reactive oxygen species; EGF, epidermal growth factor.

al., 2003). In leukemic Jurkat cells, plitidepsin also activates the Fas/CD95 receptor pathway together with JNK, possibly via the induction of cytoskeleton-mediated concentration in membrane lipid rafts of a large number of molecules including Fas ligand (Gajate et al., 2003; Gajate and Mollinedo, 2005). Plitidepsin has antiangiogenic effects in xenografted mice (Straight et al., 2006), which may result from the inhibition of vascular endothelial growth factor (VEGF) secretion and action (Broggini et al., 2003; Taraboletti et al., 2004) or to the induction of tumor or endothelial cell apoptosis (Biscardi et al., 2005). Plitidepsin blocks VEGF secretion and down-regulates VEGF mRNA in a cell line (ALL-PO) derived from a patient with acute lymphoblastic leukemia. However, plitidepsin cytotoxicity does not seem to be related to VEGF inhibition because the sensitivity of other cell lines (Reh, ALL/MIK, and TOM-1) derived from other patients with acute lymphoblastic leukemia is comparable (Erba et al., 2003).

We have recently reported that the sustained activation of JNK by plitidepsin depends on the rapid activation of Rac1 small GTPase, but not of Rho or Ras, and the subsequent down-regulation of MKP-1 phosphatase (González-Santiago et al., 2006). Rac1-JNK activation, but not MKP-1 down-regulation, is prevented by exogenous reduced GSH and compounds that restore the cellular GSH content but not by other antioxidants (González-Santiago et al., 2006). Our data thus indicate that plitidepsin induces an early oxidative stress linked to the disruption of glutathione homeostasis and activation of Rac1-JNK. However, the nature of the initial events that trigger plitidepsin signaling and effects remains unknown.

Herein, we report the first study on the binding parameters and localization of plitidepsin in human cancer cells. Using a radiolabeled plitidepsin, our results show that the compound has a moderately high-affinity receptor. Exposure to plitidepsin leads to a transient localization to the membrane, which triggers apoptosis. Later, plitidepsin predominantly accumulates in the cytosol, although almost 20% remains membrane-bound. By immunofluorescence and Western blotting analyses of subcellular fractions, we found that plitidepsin induces Rac1 translocation to the plasma membrane. Moreover, methyl-β-cyclodextrin (CD), which disrupts membrane domains such as lipid rafts by cholesterol depletion (Brown and London, 2000; Simons and Toomre, 2000), inhibits plitidepsin binding and prevents plitidepsin-induced activation of Rac1 and JNK in MDA-MB-231 cells. In addition, we report that plitidepsin-resistant HeLa cells (HeLa-R) contain less cholesterol and membrane-bound Rac1, and display reduced plitidepsin binding capacity and Rac1-JNK activation than their wild-type counterparts. Taken together, our results demonstrate an important role of cholesterol for plitidepsin binding and action, indicate that apoptosis signaling is triggered from the membrane compartment, and stress that Rac1 and JNK activation are crucial for this process.

Materials and Methods

Cell Culture. MDA-MB-231 human breast adenocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). They were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1 mM glutamine (all from Invitrogen, Paisley, UK). Wild-type HeLa cells (HeLa-wt) and HeLa-R cells showing partial resistance to plitidepsin (Losada et al., 2004) were provided by Dr. L.F. García-Fernández (Pharma Mar, Madrid, Spain) and grown in the same medium. Plitidepsin is manufactured by Pharma Mar S.A. Stock solutions were freshly prepared in dimethyl sulfoxide and diluted in the cell culture to the final concentrations indicated.

Antibodies used were: anti-JNK1, anti-c-Jun, anti-extracellular signal-regulated kinase, and anti-epidermal growth factor receptor (EGFR) from Santa Cruz Biotechnology (Santa Cruz, CA); anti-phospho-JNK1 from New England Biolabs (Ipswich, MA)/Cell Signaling Technology Inc. (Danvers, MA); anti-vimentin from Dako North America, Inc., (Carpinteria, CA); anti-Rac1 monoclonal antibody and anti-caveolin-1 rabbit polyclonal antibody from BD Transduction Laboratories (Lexington, KY); anti-transferrin receptor monoclonal antibody from Zymed Laboratories (South San Francisco, CA); Alexa Fluor 488 anti-rat IgG (H+L) from Invitrogen (Carlsbad, CA), HPR-conjugated anti-mouse IgG (H+L) from Promega (Madison, WI); and HPR-conjugated anti-rabbit IgG (H+L) from MP Biomedicals (Irvine, CA).

Subcellular Fractioning. Subcellular fractions (membrane, cytosol, cytoskeletal, nucleoplasm) were obtained by using the *Proteo-Extract Subcellular Proteome Extraction Kit* from Calbiochem (San Diego, CA) following the manufacturer's instructions. On the other hand, to prepare soluble and particulated fractions, the cell monolayers were washed in phosphate-buffered saline (PBS) and harvested for 20 min in chilled hypotonic lysis buffer (20 mM HEPES, pH 7.4). Lysates were then homogenized and passed consecutively eight times through a 9-gauge needle and 10 times through a 19-gauge needle. Homogenates were centrifuged at 3000 rpm for 5 min to pellet intact cells and nuclei. The supernatants were then spun at 100,000*g* for 30 min at 4°C in a refrigerated TL-100 ultracentrifuge to sediment particulate material. The supernatant (soluble fraction, S100) was removed, and the pellet (particulate fraction, P100) was resuspended in hypotonic lysis buffer. Protein concentrations in the fractions were determined using a colorimetric assay (Bio-Rad Laboratories, Hercules, CA).

[^{14}C]Plitidepsin. ^{14}C-labeled plitidepsin (Aplidin) ([^{14}C]APL; specific activity, 248.3 mCi/mmol; 221.6 μCi/mg, 93.06% pure by high-pressure liquid chromatography) was synthesized at Pharma Mar.

Binding Assays. For Scatchard analysis, 2–3 $\times$ 10^4 were seeded in 24-well plates. After overnight incubation, they were treated with increasing concentrations of [^{14}C]APL alone or together with a 10-fold excess of unlabeled APL as competitor for 30 min at 4°C. To study cell uptake and drug localization, cells were incubated with 450 nM [^{14}C]APL for 30 min at 37°C. The medium was then removed, and the cells were washed twice in ice-cold PBS and later incubated for 15 min at 4°C with chilled 10% TCA. Nonprecipitable material was collected and the radioactivity was counted using scintillation liquid in a β-counter. The TCA-precipitable material was solubilized in 0.2 N NaOH and 1% SDS, collected, and counted.

Detergent Extraction and Isolation of Insoluble Membranes. We followed previously described procedures (Brown and Rose, 1992; Llorente et al., 2004). HeLa cells grown to confluence in 150-mm dishes were rinsed with PBS and lysed for 20 min in 0.5 ml of 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, and 1% Triton-X-100 at 4°C. The lysate was homogenized by passing the sample through a 22-gauge needle, brought to 40% sucrose (w/w) in a final volume of 4 ml, and placed at the bottom of an 8-ml 5 to 30% linear sucrose density gradient made in the same buffer with Triton X-100. Gradients were centrifuged for 18 h at 39,000 rpm at 4°C in an SW40 rotor (Beckman Coulter, Fullerton, CA). Fractions of 1 ml were harvested from the bottom of the tube and aliquots were then subjected to SDS-PAGE under reducing conditions and analyzed by Western blotting.

Western Blotting. To study the effect of plitidepsin on the activity of JNK, cells were preincubated for 24 h in serum-free medium.

Cell protein extracts were prepared after standard procedures (Cuadrado et al., 2004). Protein extracts were electrophoresed in polyacrylamide gels and transferred to polyvinylidene difluoride (Pall Corporation, Ann Arbor, MI) membranes. The filters were washed, blocked with 5% bovine serum albumin in Tris-buffered saline (25 mM Tris, pH 7.4, 136 mM NaCl, 2.6 mM KCl, and 0.5% Tween 20), and incubated overnight at 4°C with the appropriate antibody. Blots were washed three times for 10 min in PBS + 0.1% Tween 20 and incubated with horseradish peroxidase secondary antibodies for 1 h at room temperature. Blots were developed by a peroxidase reaction using the ECL detection system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Rac1 Activity Assays. Bacterial expression of fusion proteins. The plasmid pGEX-PAK-CRIB containing the Rac1-binding domain fused to glutathione transferase (GST) was kindly provided by J.G. Collard (The Netherlands Cancer Institute). GST fusion protein was purified (from *E. coli* BL21 (DE3) harboring these plasmids) according to the method described previously (Jorge et al., 2002). For in vitro binding assays, mammalian cells were lysed in ice-cold lysis buffer (10% glycerol, 50 mM Tris pH 7.4, 1% Nonidet P-40, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of leupeptin and aprotinin) and nucleus-free supernatants were incubated with the GST-fusion protein on glutathione-Sepharose beads and analyzed as described previously (Jorge et al., 2002).

Analysis of Total Cholesterol and Plasma Membrane Cholesterol Content. Modifications were made to the method proposed by Lange and Ramos (1983) and Jacobs et al. (1997) to evaluate plasma membrane-free cholesterol in intact cells. After trypsin dissociation and washing, cells were fixed with 1% glutaraldehyde for 15 min at 4°C. The cells were then washed three times with 10 volumes of PBS, resuspended in 1 ml of PBS, and treated with 5 U/ml cholesterol oxidase (Sigma-Aldrich, St. Louis, MO) at 37°C for 1 h. Previous to lipid extraction, 20 μl of ergosterol (1 mg/ml) as an internal standard was added. Lipids were extracted twice with hexane/isopropanol (3:2), 2 ml each. Hexane fractions were collected and evaporated to dryness and cholesterol and cholestenone (derived from plasma membrane free cholesterol) were analyzed by reversed-phase high-performance liquid chromatography using a Luna-Pack 5 μm pore size C18 column (250 × 4.60 mm; Phenomenex, Torrance, CA) as described previously (Fernández-Hernando et al., 2005). Detection was performed by scanning from 200 to 320 nm. Identification of chromatographic peaks was carried out by comparing retention times and spectra with those of standards. Cholestenone was quantified by area measurements at 245 nm and cholesterol at 206 nm.

Immunofluorescence and Confocal Microscopy. Cells were rinsed twice in PBS, fixed in 3.7% *para*-formaldehyde for 10 min at room temperature (RT) and subsequently permeabilized with 0.5% Triton X-100 in PBS for 20 min at RT and then incubated in 0.1 M glycine for 30 min, 1% bovine serum albumin for 15 min, and 0.01% Tween 20 (all in PBS) for 5 min. For immunolabeling, cells were rinsed three times in PBS containing 0.05% Tween 20 (PBS containing 0.05% Tween 20), incubated for 2 h at RT with anti-Rac1 antibody, washed in PBS containing 0.05% Tween 20, and incubated for 45 min with the appropriate secondary antibody conjugated to Alexa Fluor 488. For lipid raft (ganglioside GM1) staining, before the fixation samples were labeled with Vybrant Lipid Raft Labeling Kit (Invitrogen) following the manufacturer's recommendations, and then fixed and permeabilized as indicated above for subsequent staining with anti-Rac1 antibody. In all cases samples were counterstained with 4,6-diamidino-2-phenylindole diluted in PBS (Sigma) for nucleus staining. Finally, the coverslips were mounted in VectaShield (Vector Laboratories, Peterborough, UK) and sealed with nail polish. Confocal microscopy was performed with a Bio-Rad MRC-1024 laser scanning microscope, equipped with an Axiovert

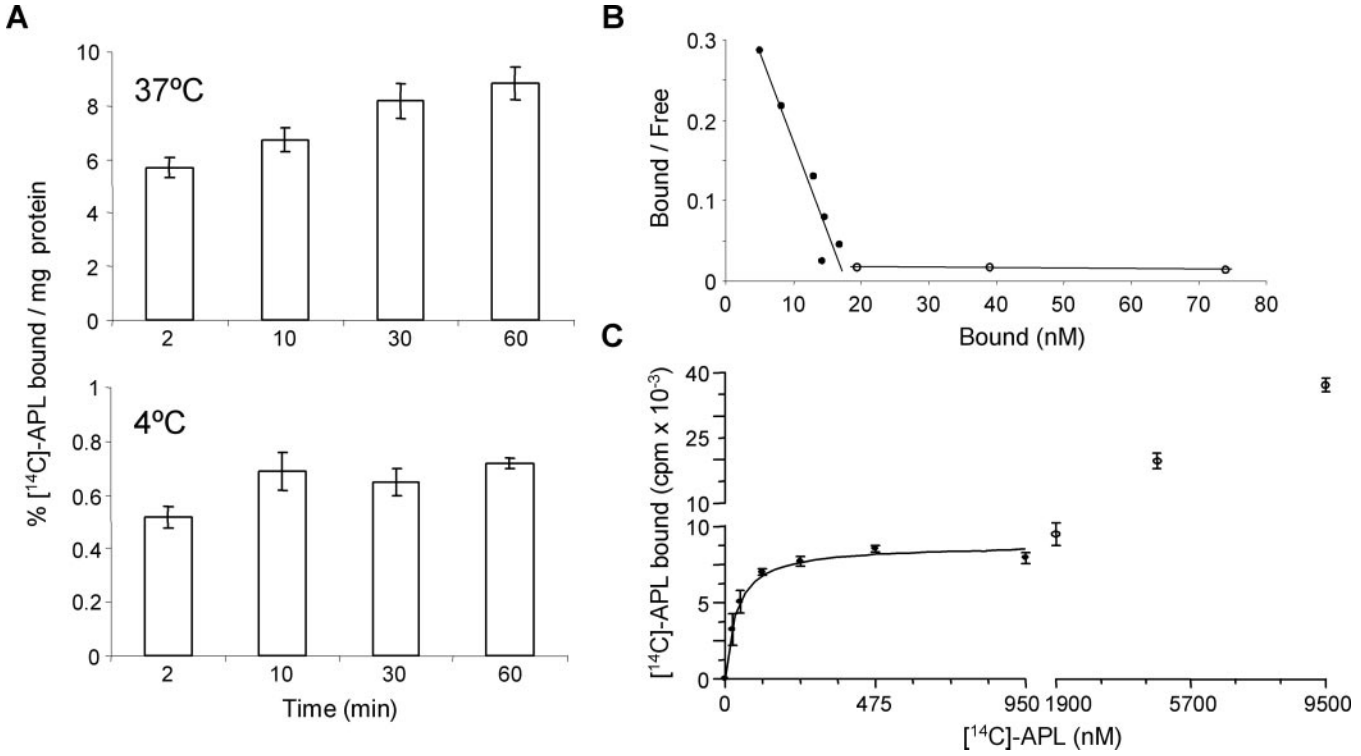

Fig. 1. Cell binding properties of plitidepsin. Specific binding was calculated by subtracting from absolute values the nonspecific binding determined by parallel incubations with 5-fold molar excess unlabeled plitidepsin. Data (percentage of drug bound) correspond to the mean ± S.D. from three independent experiments performed in triplicate. A, MDA-MB-231 cells were incubated at either 37°C (top) or 4°C (bottom) with 450 nM [^{14}C]APL for the indicated times. Cellular uptake of the drug was estimated as described under *Materials and Methods*. Note the different scales. B, Scatchard analysis of plitidepsin binding. Cells were incubated with the indicated range of concentrations of [^{14}C]APL for 30 min. ●, moderately high-affinity binding site; ○, low-affinity high capacity binding sites. C, saturation analysis of plitidepsin binding. Note the changes in scale in both axes.

100 invert microscope (Zeiss, Welwyn Garden City, UK) at excitation wavelengths of 488 nm (for fluorescein isothiocyanate), 543 nm (for Alexa), and 351/364 nm (for 4,6-diamidino-2-phenylindole). Each channel was recorded independently, and pseudocolor images were generated and superimposed. Images were processed with the use of Photoshop 7.0 software (Adobe Systems, Mountain View, CA).

Statistical Analysis. The results are expressed as mean ± S.D. or S.E.M. Statistical comparisons between groups were performed using the Student's *t* test.

Results

Cellular Binding of Plitidepsin. To examine the cellular binding and localization of plitidepsin, we used [^{14}C]APL. First, we analyzed drug distribution in trichloroacetic acid (TCA)-precipitable (bound to macromolecules) and TCA-soluble (free) fractions after addition to MDA-MB-231 cells. At 37°C, the amount of drug present in TCA-precipitable material increased from around 6% 2 min after addition to around 9% 60 min later (Fig. 1A, top). The uptake in the same period at 4°C was only 0.5 to 0.7% (Fig. 1A, bottom). To determine the cellular affinity for plitidepsin, we performed Scatchard analyses. Results revealed a moderately high-affinity binding site (K_d of 44.8 ± 3.1 nM) (Fig. 1B, ●) and low-affinity/high-capacity sites (Fig. 1B, ○). Saturation binding experiments confirmed these binding parameters (K_d of 35.8 ± 4.8 nM) and showed that the high-affinity binding site was saturable with relatively low doses of drug, whereas the low-affinity sites were nonsaturable (Fig. 1C, ● and ○, respectively).

To examine the subcellular distribution of [^{14}C]APL, we used fractioning procedures (see *Materials and Methods*) that produced highly enriched cytosolic, membrane, nucleoplasmic, and cytoskeletal fractions, as confirmed by Western blotting using antibodies against Erk, epidermal growth factor receptor, c-Jun, and vimentin (Fig. 2A, left). At 37°C, most [^{14}C]APL (around 80%) was located in the cytosol 30 min after the addition, and 19% in the membrane fraction (Fig. 2A, top right). Similar results were obtained when particulate (P100) and soluble (S100) fractions were obtained by ultracentrifugation (Fig. 2A, right, inset). In contrast, at 4°C (again, 10-fold lower binding was found) 55% [^{14}C]APL was membrane-bound, attributable to the rigidity of plasma membrane and reduction of active transport at this temperature, whereas 40% is cytosolic (Fig. 2A, bottom right). No significant amount of drug locates in either the nucleoplasm or cytoskeleton fractions at any temperature.

Time course experiments showed that 2 min after addition, the drug was evenly distributed between membrane and cytosolic fractions and confirmed that a greater amount (70–80%) subsequently locates in the cytosol, whereas around

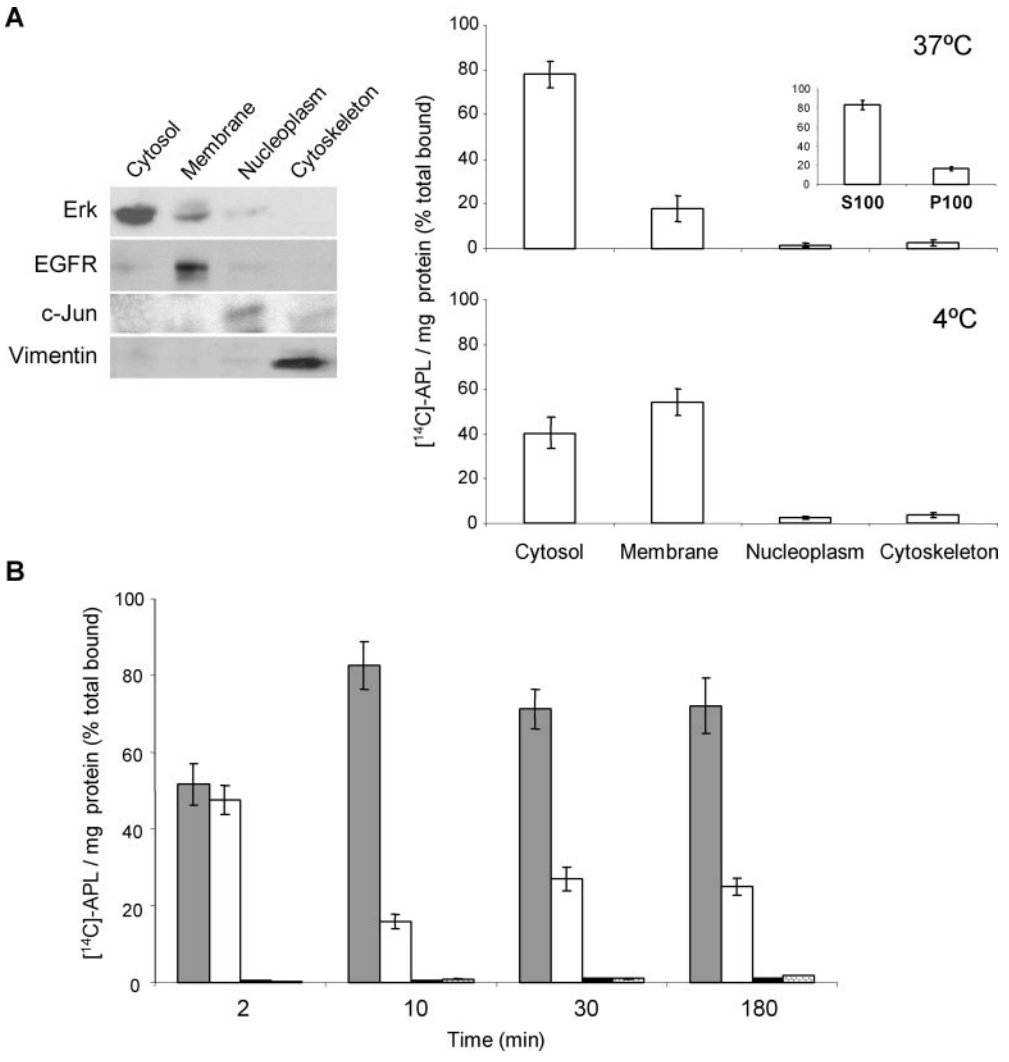

Fig. 2. Subcellular localization of plitidepsin. A, left, Western blot analysis of MDA-MB-231 cell fractions obtained as described under *Materials and Methods*. Antibodies against Erk, epidermal growth factor receptor (EGFR), c-Jun, and Vimentin were used to analyze purity of fractions. Right shows the distribution of [^{14}C]APL in the different fractions at 37°C (top) or 4°C (bottom). Inset shows drug distribution in soluble (S100) and particulate (P100) fractions prepared as described under *Materials and Methods*. B, distribution of [^{14}C]APL in the cytosol (gray column), membrane (white column), nucleoplasm (black), and cytoskeleton (dotted column) fractions at different times after addition to MDA-MB-231 cells. Data (percentage of total drug bound present in each fraction) correspond to the mean ± S.D. from three independent experiments performed in triplicate.

20% remains membrane-bound (Fig. 2B). Short pulse (1 h) treatment with the drug at either 37°C or 4°C induced strong cytotoxicity (around 85% of that induced by continuous treatment) and 5 min of exposure to the drug caused half-maximum cell death at 48 h thereafter (data not shown).

Depletion of Plasma Membrane Cholesterol Inhibits Plitidepsin Action. Given that cholesterol is required for the integrity and signaling from membrane domains such as lipid rafts and that these domains are crucial for Rac activity (del Pozo et al., 2000, 2004), we examined whether it could affect plitidepsin action. Total cholesterol content in MDA-MB-231 cells was 47.7 μg/mg protein, approximately 50% of which was localized in the plasma membrane. Treatment with CD removed practically all the free cholesterol from the plasma membrane, decreasing the cell content to 23.3 μg/mg protein (49%). Pretreatment with CD significantly decreased [^{14}C]APL binding, whereas exogenously added cholesterol (Chol; 16 μg/ml) reverted this effect (Fig. 3A, left). These results were confirmed using P100 and S100 fractions (Fig. 3A, right). In line with this, CD inhibited in a cholesterol-dependent fashion the activation of Rac1 GTPase by plitidepsin (Fig. 3B). In agreement with its dependence on Rac1 (González-Santiago et al., 2006), JNK activation by the drug was inhibited by CD pretreatment (Fig. 3B). Again, exogenous cholesterol rescued this inhibition (Fig. 3B). Likewise, CD abrogated the activation of p38 MAPK by plitidepsin (data not shown).

Rac1 GTPase activity is dependent on its association with membranes (del Pozo et al., 2004). Immunofluorescence analysis followed by confocal microscopy revealed that plitidepsin increased plasma membrane localization of Rac1 as early as 5 min after treatment, an effect that was more generalized at 15 min and lasted in a subset of cells for at least 60 min (Fig. 4A). Epidermal growth factor was used as a positive control for Rac1 redistribution. In addition, the use of an antibody against the lipid raft ganglioside GM1 revealed a substantial localization of Rac1 in these membrane domains upon plitidepsin treatment (Fig. 4B). Western blotting analysis of membrane fractions confirmed that plitidepsin transiently increased membrane-bound Rac1 (Fig. 4C). CD prevented this effect, whereas simultaneous treatment with CD and exogenous cholesterol delayed Rac1 membrane accumulation.

Plitidepsin-Resistant HeLa-R Cells Have Lower Membrane Cholesterol and Show Reduced Plitidepsin Binding and Rac1 Activation. Plitidepsin-resistant Hela-R cells show reduced JNK activation upon drug treatment (Losada et al., 2004). To examine whether their partial resistance could be a consequence of diminished drug uptake, we performed binding assays using [^{14}C]APL. HeLa-R cells incorporated 5-fold less drug into TCA-precipitable material than HeLa-wt cells (Fig. 5A). Saturation binding experiments showed that HeLa-R cells (squares) contain around 7.5-fold fewer moderately high-affinity binding sites (K_d, 79.5 ± 2.5 nM) than HeLa-wt cells (circles) (K_d, 37.7 ± 8.2 nM) (Fig. 5B, note the change in scale). Like MDA-MB-231, both HeLa cell types had nonsaturable, low-affinity binding sites. In HeLa-wt cells, the total cholesterol content was similar to that in MDA-MB-231 cells and, again, approximately one half-localized along the plasma membrane (Table

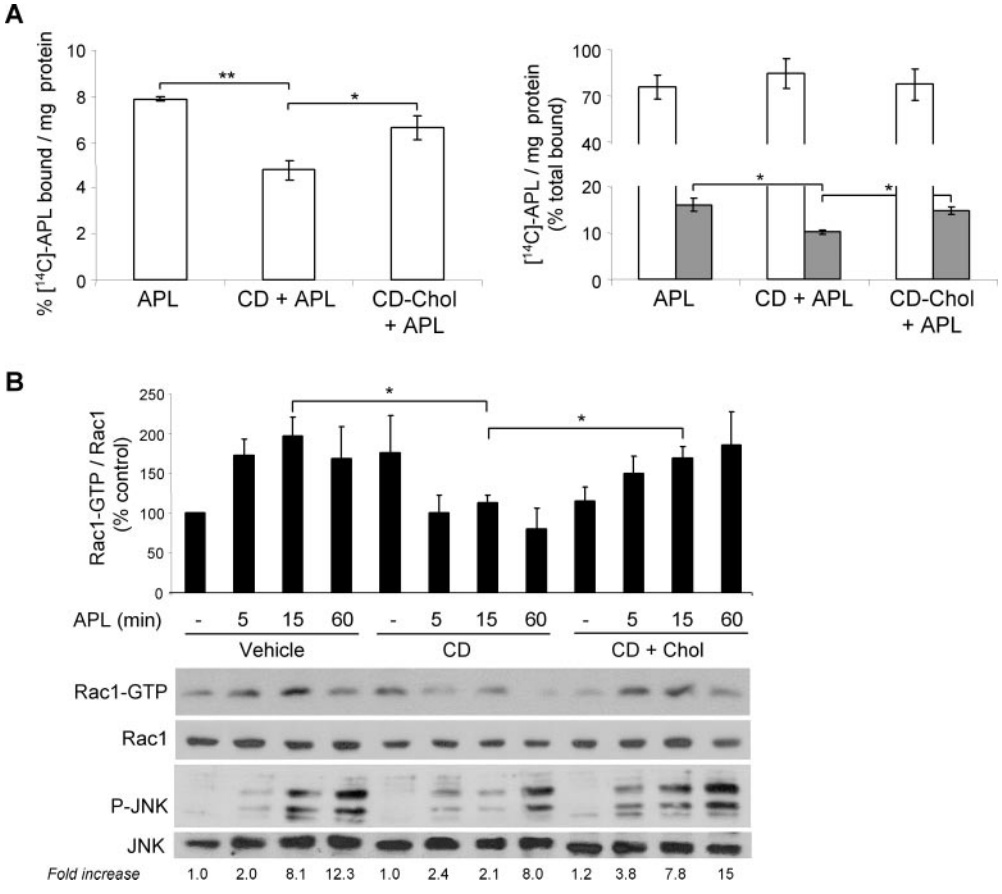

Fig. 3. Depletion of membrane cholesterol reduces plitidepsin binding and action. A, cholesterol depletion decreases membrane-bound plitidepsin. MDA-MB-231 cells were treated for 30 min with 450 nM [^{14}C]APL alone (APL) or after 1-h pretreatment with 10 mM methyl-β-cyclodextrin (CD + APL) or 5 mM methyl-β-cyclodextrin and 16 μg/ml cholesterol (CD-Chol + APL), and the amount of drug incorporated into TCA-precipitable material was estimated as described under *Materials and Methods* in either whole-cell extracts (left) or separate S100 (white columns) and P100 (gray columns) fractions (right). Data (percentage of total bound drug) correspond to mean ± S.E.M. from three independent experiments performed in triplicate. Statistical comparison between groups indicated: *, $p < 0.05$; **, $p < 0.01$. B, cholesterol depletion inhibits Rac1 and JNK activation by plitidepsin. Cells were pretreated for 1 h with vehicle (−), 10 mM CD, or 5 mM CD and 16 μg/ml cholesterol (CD + Chol) before addition of 450 nM APL. Rac1 and JNK activation were analyzed at the indicated times after APL addition by estimating Rac1-GTP and phospho-JNK levels, respectively, as described under *Materials and Methods*. Total Rac1 and JNK levels were analyzed by Western blots for normalization. Histograms represent the Rac-GTP/total Rac ratios as percentage of control, and correspond to mean ± S.E.M. from five independent experiments; *, $p < 0.05$.

1). Total and plasma membrane cholesterol in HeLa-R cells were lower (71% and 67%, respectively) than those in HeLa-wt cells (Table 1). HeLa-R cells, which show no JNK activation in response to plitidepsin (Losada et al., 2004; data not shown), consistently did not respond to drug treatment with Rac1 activation (Fig. 5C). These results cannot be explained by an intrinsic defect of HeLa-R cells because they showed stronger activation of Rac1 (and JNK) in response to epidermal growth factor than HeLa-wt (Fig. 5C). Western blot analysis showed that HeLa-R cells have a similar total Rac1 protein content to HeLa-wt cells, but a lower proportion of membrane-bound Rac1 (Fig. 5D). Together with their lower cholesterol levels, this difference may explain the partial resistance of HeLa-R cells to plitidepsin.

We have shown previously that Rac1 down-regulation by siRNA or inhibition by a specific inhibitor decreases JNK activation and apoptosis induction by plitidepsin (González-Santiago et al., 2006). Two additional experiments were performed to further confirm this: first, we showed that overexpression of Rac1 in HeLa cells enhances the apoptotic action of plitidepsin (Supplementary Fig. 1); second, a transfected (hemagglutinin-tagged) dominant-negative Rac1 mutant (HA-N17-Rac1) diminished JNK activation by the drug (Supplementary Fig. 2). This effect was less strong and more cytotoxic than those found using siRac1 or the Rac1 inhibitor, probably because dominant-negative Rac1 can compete for guanine-exchange factors (GEFs) some of which are shared by other small GTPases (Feig, 1999). Finally, to examine

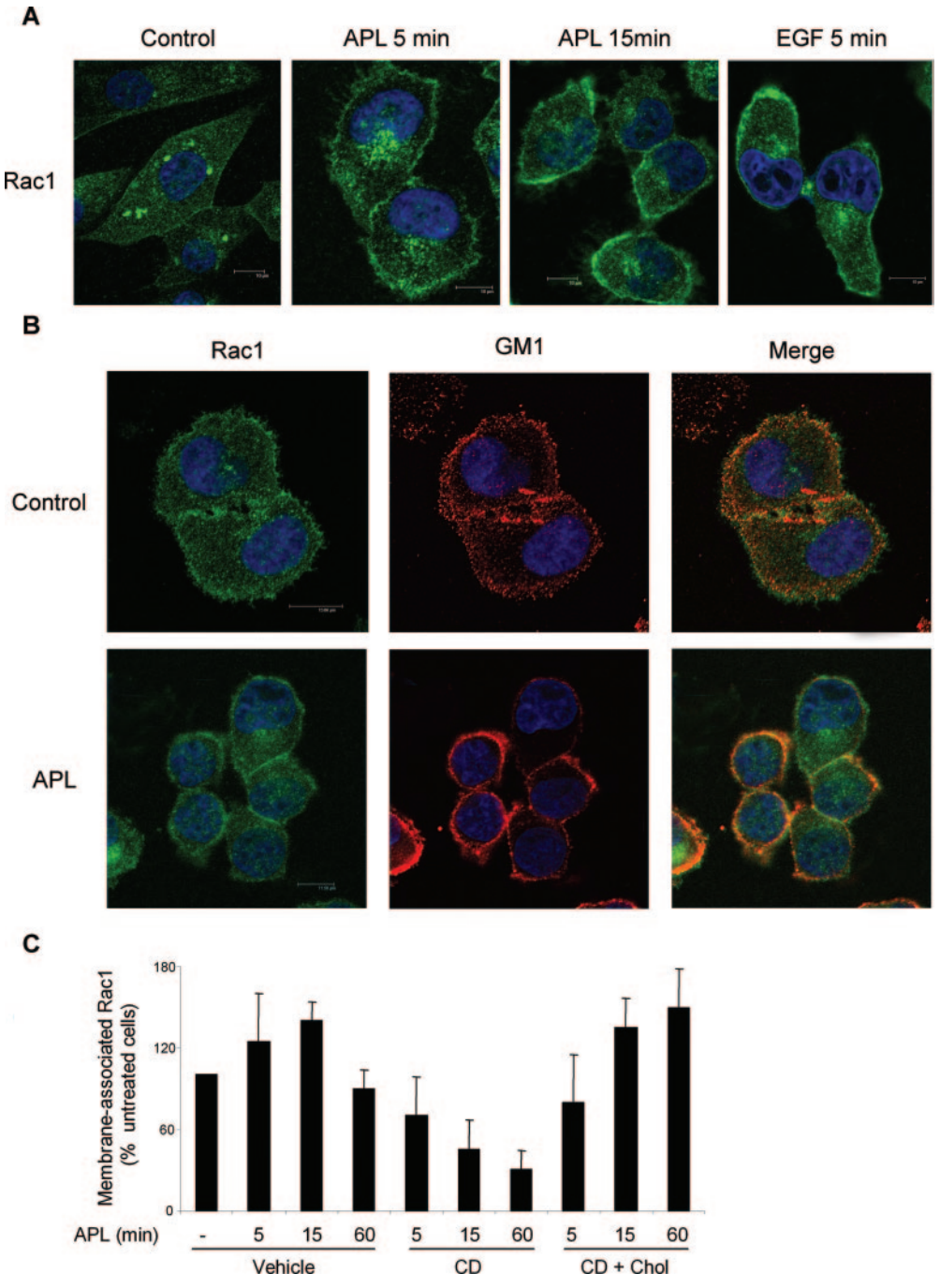

Fig. 4. Plitidepsin induces Rac1 relocalization to the plasma membrane. A, Rac1 protein expression in MDA-MB-231 cells treated with 450 nM APL or 20 ng/ml EGF for the indicated times, or left untreated (Control). Immunofluorescence and confocal microscopy analysis were performed as described under *Materials and Methods*. Bars, 10 μm. B, colocalization of Rac1 protein and GM1 ganglioside at the plasma membrane of cells treated with 450 nM APL or vehicle (Control) for 15 min. Bars, 15.9 μm (upper images); 11.6 μm (lower images). C, quantification of membrane-bound Rac1 protein as estimated by Western blot analysis of membrane fraction of cells pretreated with vehicle (−), 10 mM CD or 5 mM CD and 16 μg/ml cholesterol (CD + Chol) before addition of 450 nM APL (mean values obtained in two independent experiments).

whether also in HeLa cells plitidepsin changes the localization of Rac1 in cholesterol-rich membrane domains, we performed centrifugation to equilibrium in linear sucrose density gradients. Similarly to epidermal growth factor (EGF) used as positive control, plitidepsin increased the amount of Rac1 detected in fractions corresponding to lipid rafts, which lack transferrin receptor and contain high levels of caveolin-1 (Fig. 6, A and B).

Discussion

In this study, we report the presence of a moderately high-affinity receptor of plitidepsin, and of additional low-affinity high-capacity binding site(s), in human cancer cells. The analyses of binding kinetics at 37°C and 4°C and of subcellular fractions indicate that plitidepsin first binds to a membrane receptor and then distributes to the cytosol, although around 20% remains at the membrane. In addition, our results show the importance of Rac1 activation and translocation to cholesterol-rich plasma membrane domains for plitidepsin action. These crucial effects are inhibited by exogenous GSH, which in line with previous results (González-Santiago et al., 2006), indicates that plitidepsin action depends on a very early oxidative process.

The finding that short plitidepsin treatments at 4°C are highly cytotoxic suggests that the apoptosis is rapidly triggered upon plitidepsin binding to the plasma membrane. At this temperature, cellular binding is 10-fold lower than at 37°C. However, 40% [^{14}C]APL is found in the cytosol at 4°C, suggesting that the increase in membrane rigidity at low temperature does not totally preclude drug entry, probably because of its chemical nature (hydrophobicity, three-dimensional structure). Therefore, additional molecular mechanisms triggered by the internal receptor(s) may contribute to cell death. For instance, Src (and also EGFR) activation are secondary to JNK activation and contribute, like p38MAPK, to plitidepsin cytotoxicity, although none of them are strictly necessary (Cuadrado et al., 2004). Likewise, plitidepsin induces expression of several genes of the AP-1 (c-*JUN,* c-*FOS*. . .) and NFκB (p65/*REL*. . .) families (Cuadrado et al., 2004), but gene expression is not required for the apoptotic process (González-Santiago et al., 2006).

The Rho family of small GTPases (Rac, Rho, Cdc42), like the Ras family, function as binary switches in signaling pathways controlling cell proliferation, survival, and differentiation by cycling between the inactive GDP-bound and the active GTP-bound states. Rho GTPases are activated through

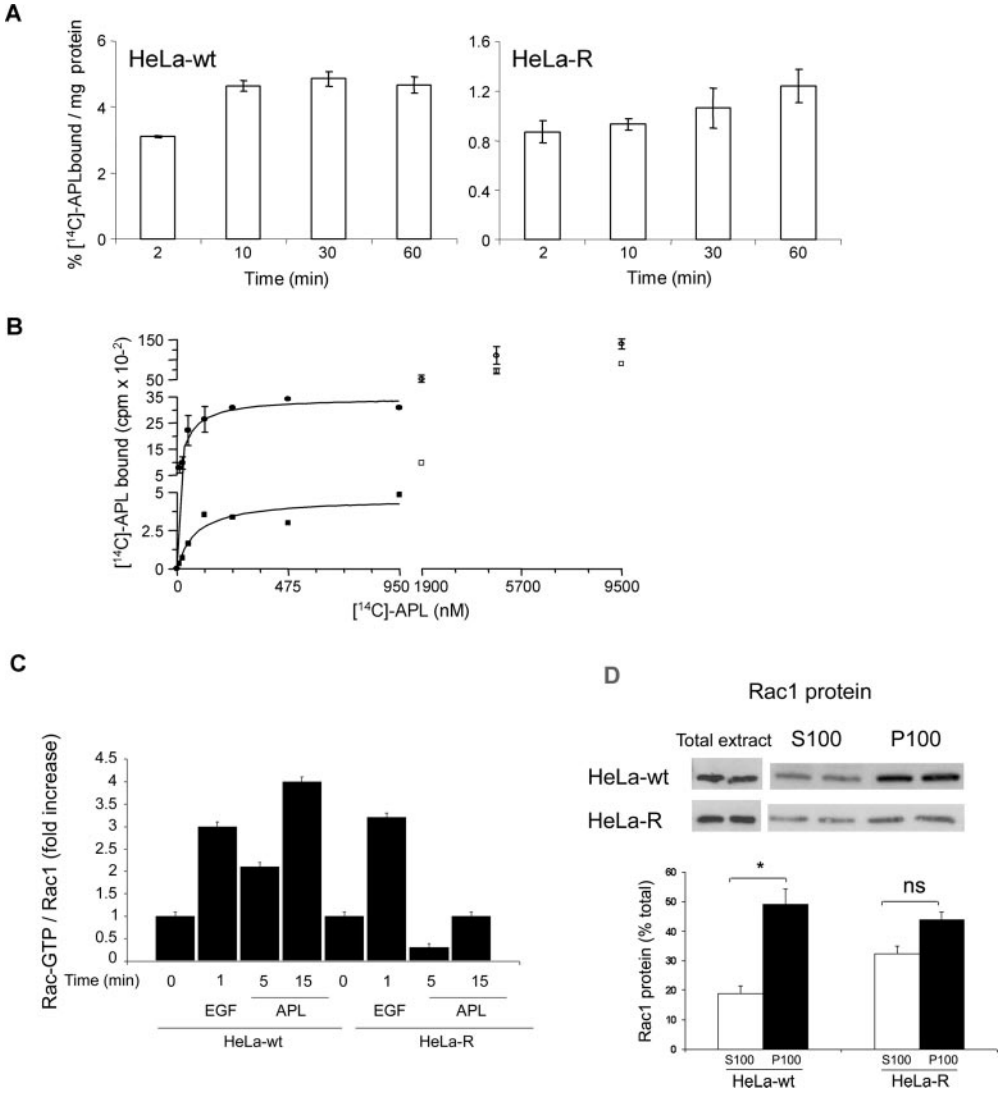

Fig. 5. Plitidepsin-resistant HeLa-R cells have reduced drug binding sites and show no Rac1 activation. A and B, specific binding was calculated as explained in legend to Fig. 1. Data (percentage of bound drug) correspond to the mean ± S.D. from three independent experiments performed in triplicate. A, comparative analysis of [^{14}C]APL binding to HeLa-wt and HeLa-R cells estimated by the incorporation into TCA-precipitable material at different times after addition measured as described under *Materials and Methods*. Note the different scale in the panels. B, saturation analysis of plitidepsin binding to HeLa-wt (circles) and HeLa-R (squares) cells. Closed symbols, moderately high-affinity binding sites; open symbols, nonsaturable low-affinity sites. Note the change in scale in both axes. C, Rac1 activation was analyzed at the indicated times after APL (150 nM) addition by estimating Rac-GTP levels as described under *Materials and Methods*. Total Rac1 content was analyzed by Western blots for normalization. EGF (100 ng/ml) was used as positive control. Fold-increase values were calculated from three independent experiments. D, Western blot analysis of Rac1 content in total cell extracts and soluble/S100 and particulated/P100 fractions of HeLa-wt and HeLa-R cells. A representative experiment is shown (top). Data correspond to the mean ± S.D. from three independent experiments performed in duplicate (down).

interaction with a number of GEFs after activation of cellular receptors for mitogens, cytokines, G-protein-coupled receptors, or adhesion receptors (Zheng, 2001). GEFs catalyze the exchange of bound GDP with cytosolic GTP. Negative regulation of Rho GTPases is carried out by Rho GTPase-activating proteins and Rho-GDP-dissociation inhibitors. It is remarkable that Rho and Ras GTPases are also activated by oxidative stress, and thus a redox-active motif has recently been characterized in several families of GTPases (Turcotte et al., 2003; Heo and Campbell, 2005).

Our results, showing that Rac1 membrane translocation and activation by plitidepsin are related to an oxidative stress and are dependent on cholesterol, are consistent with the literature. For instance, Rac1 membrane translocation and activation are stimulated by H_2O_2 in human smooth muscle cells, and this effect is blocked by simvastatin, an inhibitor of HMG-CoA reductase that decreases cholesterol synthesis (Nègre-Aminou et al., 2002). In addition, Rac activity generates ROS in several signaling pathways by different mechanisms. One of them is the activation of an NAD(P)H oxidase in neutrophils (Bokoch and Knaus, 2003; Mizrahi et al., 2005) or of another uncharacterized oxidase in most cell types. Rac also mediates ROS production by growth

TABLE 1

Cholesterol content of HeLa-wt and HeLa-R cells

Membrane cholesterol corresponds to cholestenone formed by the action of cholesterol oxidase on intact cells. Results are expressed as micrograms of cholesterol per milligram of cell protein. Data are presented as means ± S.E.M.

HeLa Cells	HeLa-wt	HeLa-R
Total cholesterol	41.5 ± 0.8	29.5 ± 2.3**
Membrane cholesterol	24.9 ± 2.3	16.8 ± 0.7*

*, $P < 0.05$; **, $P < 0.01$, statistical comparisons by Student's t test.

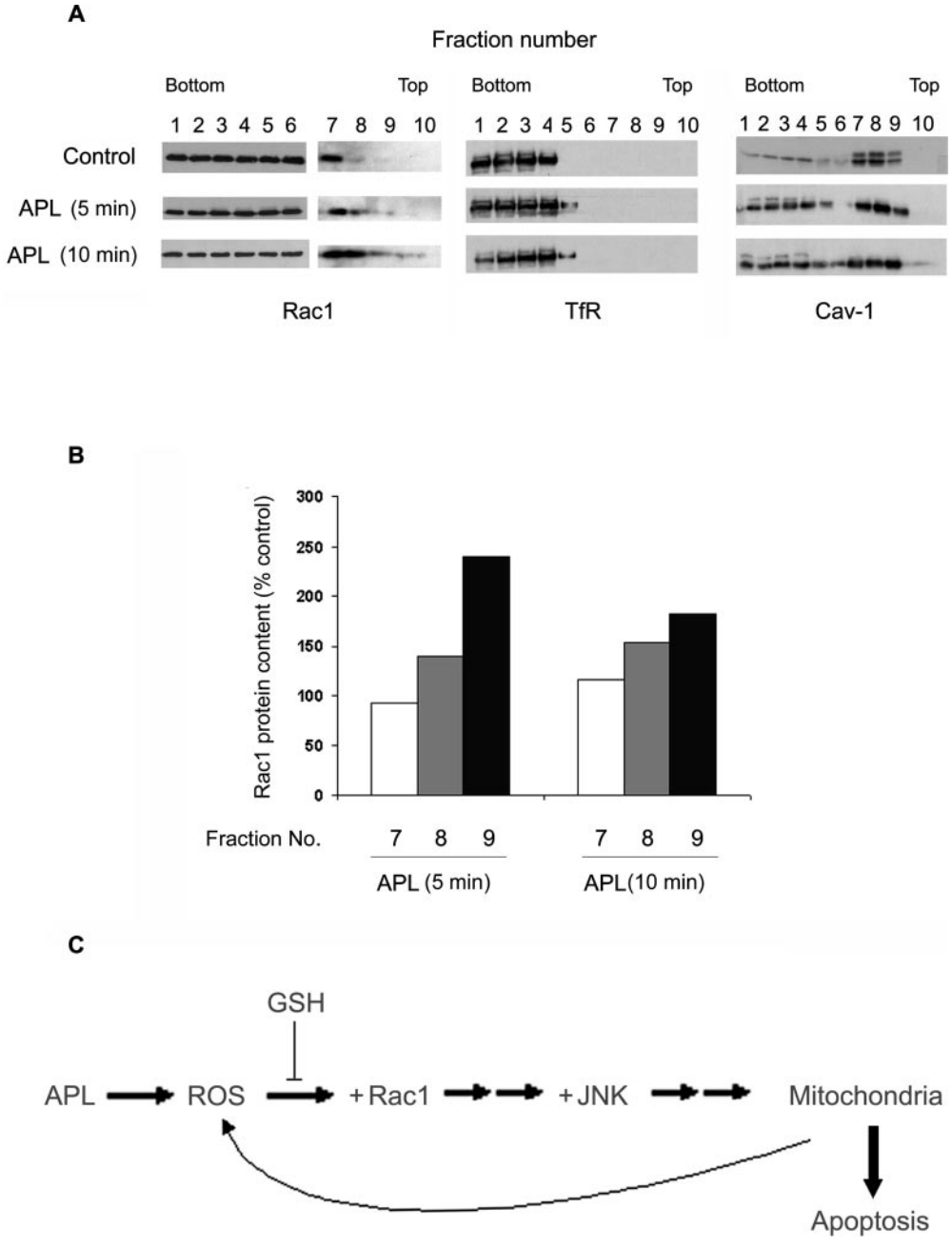

Fig. 6. Plitidepsin increases Rac1 localization at cholesterol-rich membrane domains. A, HeLa-wt cells were treated with plitidepsin (450 nM) for 5 or 10 min and then extracted with Triton X-100 at 4°C and subjected to centrifugation to equilibrium in sucrose density gradients. Aliquots from each fraction were analyzed by Western blotting with antibodies specific to Rac1. As controls for the fractioning procedure, we used antibodies to caveolin-1 (cav-1) and to transferrin receptor (TfR). Fractions 1 to 4 represent the 40% sucrose layer and contain the bulk of cellular membranes and cytosolic proteins (also most of Rac1; exposure time for Rac1, 10 s), and fractions 5 to 12 represent the 5 to 30% sucrose layer that contain rafts (exposure time for Rac1, 5 min). Fractions 11 and 12 are not shown because they contained no Rac1, TfR, or cav-1 signals. B, quantification of the level of Rac1 protein in fractions 7 to 9 of the sucrose gradients. C, scheme of the proposed positive regulatory feed-back mechanism of ROS generation leading to apoptosis of plitidepsin-treated cells.

factors such platelet-derived growth factor and EGF (Joyce et al., 1999; Page et al., 1999) and by noncytotoxic TNF-α signaling (Woo et al., 2000). In addition, Rac is necessary for integrin-dependent increase in H_2O_2 production (Werner and Werb, 2002). The rapid activation of Rac by plitidepsin is inhibited by GSH, suggesting the induction of an early oxidative stress. Moreover, plitidepsin progressively increases ROS in MDA-MB-231 cells and disrupts mitochondrial membrane potential and function, which is in turn a source of ROS (González-Santiago et al., 2006). It is thus conceivable that a positive feedback regulatory loop of ROS production leads to apoptosis of plitidepsin-treated cells (Fig. 6C).

Previous data showed that Rac1 down-regulation by siRNA inhibits, but does not completely abrogate, JNK activation and cytotoxicity by plitidepsin. However, total Rac silencing could not be obtained, and because Rac-deficient cells are not viable, others mediators of the activation of JNK by plitidepsin cannot be ruled out (González-Santiago et al., 2006). Activated, GTP-bound Rac interacts with a series of effector targets, some of which, such as p21-activated protein kinase or mitogen-activated protein kinase kinase kinase can in turn activate JNK. Currently the mechanism of JNK activation by plitidepsin and associated JNK substrates are unclear. The finding that c-*jun*–deficient MEFs show comparable sensitivity to the drug to their wild-type counterparts argues against a role for c-JUN (Cuadrado et al., 2004). The mechanism of induction of apoptosis by JNK is elusive. Several Bcl-2 family members as well as interacting proteins such as 14-3-3 have been proposed as JNK targets and so are candidates to mediate plitidepsin-induced apoptosis but the exact molecular basis remains unknown (Schroeter et al., 2003; Sunayama et al., 2005). This hypothesis is also supported by the reported modulation of mitochondrial function by Rac1 in rabbit synovial fibroblasts through the control of the function of Bcl-2 family members (Werner and Werb, 2002).

Integrins regulate Rac1 targeting to cholesterol-rich plasma membrane domains and its coupling to downstream effectors such as p21-activated kinase (del Pozo et al., 2000, 2004). Thus, plitidepsin could putatively activate Rac1 through effects on integrin expression and/or signaling. Examination of this possibility would require extensive research, owing to the large number of integrin heterodimers and interacting proteins.

Our work comparing the effects of plitidepsin on HeLa-wt and HeLa-R cells supports the involvement of Rac1 in the mechanism of action of plitidepsin. The hypothesis of cholesterol dependence is also strongly supported, in that the lower cholesterol content of HeLa-R cells may be responsible for their partial resistance to plitidepsin. This resistance would result from decreased binding of the drug to its moderately high affinity receptor and the significant reduction of Rac1-JNK activation. In contrast, those molecular events linked to binding to the internal receptor may remain active in HeLa-R cells. This system may be used in the search for crucial binding and target proteins of plitidepsin. Thus, we have begun a proteomic analysis of membrane and cytosolic fractions aimed to identify proteins differentially expressed in the two cell types. A putative limitation of this approach, however, is the lower cholesterol content of HeLa-R cells, which may affect the cellular response to plitidepsin independently of changes in protein expression. The lower content of membrane-bound Rac1 and cholesterol of HeLa-R cells may be at least partially responsible for their reduced sensitivity to the drug. Given the high proapoptotic activity of plitidepsin, the identification of the moderately high affinity receptor here described could be of pharmacological importance.

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Address correspondence to: Prof. Alberto Muñoz, Instituto de Investigaciones Biomédicas "Alberto Sols," Arturo Duperier, 4, E-28029 Madrid, Spain. E-mail: amunoz@iib.uam.es