Membrane association of Ras proteins is crucial for their signaling and transforming activities (17); thus, the Ras farnesyl group has been targeted therapeutically (22, 23). Among Ras proteins, K-Ras has the highest incidence of constitutively active mutations in human tumors (e.g. pancreatic, colorectal, and lung tumors) (2, 24). Thus, specific interference with its signaling via perturbation of its unique electrostatic interactions with the membrane holds the potential for selective intervention. Chlorpromazine (CPZ)\(^4\) is a cationic amphiphilic phenothiazine employed as a neuroleptic drug in treatments of schizophrenia and terminal cancer symptom alleviation (25). In cultured cells, CPZ markedly affects the form and composition of the plasma membrane. In erythrocytes, the hydrophobic part of CPZ inserts into the inner membrane leaflet, whereas its cationic portion interacts electrostatically with negatively charged phospholipids in general and phosphoinositides in particular. This alters the chemo-physical properties of the membrane, causing stomatocystosis and redistribution of different Ras proteins are non-redundant (reviewed in Refs. 4–6). A plausible mechanism that could give rise to some of these differences is the distinct membrane anchorage of each Ras isoform, mediated by their variable C termini, termed "the hypervariable regions." These regions also regulate intracellular trafficking, endomembrane targeting, and microdomain association of Ras isoforms (7–15). The membrane tethering of Ras proteins is mediated by at least two signals. They share a conserved C-terminal CAAX motif that undergoes farnesylation (16). However, a second signal is required; the hypervariable regions of H-Ras, N-Ras, and K-Ras4A contain one or two cysteines that undergo palmitoylation, whereas in K-Ras4B (designated K-Ras hereafter) the second signal is a 6-lysine polybasic motif that interacts electrostatically with negatively charged phospholipids in the internal membrane leaflet (15, 17–21). Recently, phosphorylation of residues in the immediate vicinity of the polylysine stretch (Ser\(^181\), and to a lesser degree Ser\(^171\) and Thr\(^183\)), was shown to modulate electrostatic-based interactions of K-Ras with the plasma membrane (13).

Membrane association of Ras proteins is crucial for their signaling and transforming activities (17); thus, the Ras farnesyl group has been targeted therapeutically (22, 23). Among Ras proteins, K-Ras has the highest incidence of constitutively active mutations in human tumors (e.g. pancreatic, colorectal, and lung tumors) (2, 24). Thus, specific interference with its signaling via perturbation of its unique electrostatic interactions with the membrane holds the potential for selective intervention. Chlorpromazine (CPZ)\(^4\) is a cationic amphiphilic phenothiazine employed as a neuroleptic drug in treatments of schizophrenia and terminal cancer symptom alleviation (25). In cultured cells, CPZ markedly affects the form and composition of the plasma membrane. In erythrocytes, the hydrophobic part of CPZ inserts into the inner membrane leaflet, whereas its cationic portion interacts electrostatically with negatively charged phospholipids in general and phosphoinositides in particular. This alters the chemo-physical properties of the membrane, causing stomatocystosis and redistribution of different Ras proteins are non-redundant (reviewed in Refs. 4–6). A plausible mechanism that could give rise to some of these differences is the distinct membrane anchorage of each Ras isoform, mediated by their variable C termini, termed "the hypervariable regions." These regions also regulate intracellular trafficking, endomembrane targeting, and microdomain association of Ras isoforms (7–15). The membrane tethering of Ras proteins is mediated by at least two signals. They share a conserved C-terminal CAAX motif that undergoes farnesylation (16). However, a second signal is required; the hypervariable regions of H-Ras, N-Ras, and K-Ras4A contain one or two cysteines that undergo palmitoylation, whereas in K-Ras4B (designated K-Ras hereafter) the second signal is a 6-lysine polybasic motif that interacts electrostatically with negatively charged phospholipids in the internal membrane leaflet (15, 17–21). Recently, phosphorylation of residues in the immediate vicinity of the polylysine stretch (Ser\(^181\), and to a lesser degree Ser\(^171\) and Thr\(^183\)), was shown to modulate electrostatic-based interactions of K-Ras with the plasma membrane (13).
Modulation of K-Ras by Chlorpromazine

phospholipids between the inner and outer leaflets (26). Furthermore, CPZ inhibits calmodulin, which regulates the availability of phosphoinositides via membrane recruitment of potential sequestrators of phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) such as myristoylated alanine-rich C kinase substrate (MARCKS) (27, 28). Accordingly, CPZ inhibits clathrin-mediated endocytosis (29) by abrogating the PI(4,5)P$_2$-dependent membrane recruitment of the AP2 adaptor complex. Phenothiazines were also shown to inhibit proliferation and cause apoptosis of tumor cell lines, to abrogate differentiation, and to reverse multidrug resistance (30–32). In accord with its ability to specifically dislodge oncogenic K-Ras(G12V) from the plasma membrane. Our data demonstrate that CPZ perturbs the plasma-membrane association of GFP-tagged K-Ras(G12V) or Rac1(G12V), which have a contribution of electrostatic interactions to their membrane targeting, without affecting H-Ras. Depending on the cell type, this led to a significant increase in the level of GFP-K-Ras(G12V) in the cytoplasm and/or in specific internal membranes. Thus, in Rat-1 cells CPZ shifted part of the K-Ras(G12V) population to mitochondria and induced apoptosis, whereas in Panc-1 cells CPZ induced cell-cycle arrest and no localization to mitochondria. Importantly, in Panc-1 cells stably expressing GFP-K-Ras(G12V), CPZ inhibited cell migration and colony formation in soft agar. These results suggest distinct mechanisms for the specific interference of CPZ with K-Ras signaling and oncogenic potential, based on the perturbation of its membrane interactions.

EXPERIMENTAL PROCEDURES

Materials and Plasmids—CPZ hydrochloride, W13, methylene blue, mouse anti-lactate dehydrogenase, and mouse anti-active caspase 3 antibodies were from Sigma. Mouse Pan anti-Ras antibody (Ab-3) was from Calbiochem. Rabbit anti-caveolin 1 was from Cell Signaling Biotechnology, Annexin V-biotin from R&D Systems, Cy3-streptavidin from Jackson ImmunoResearch, Alexa 546-transferrin from Invitrogen-Molecular Probes, and peroxidase goat anti-mouse or anti-rabbit IgG from Dianova. pEYFP-C1 expression vectors for YFP-K-Ras(G12V), YFP-K-Ras(G12V,S181E), YFP-K-Ras(G12V, S181A), and YFP-K-Ras(G12V,S171A,S181A,T183A) (13) were a generous gift from Dr. M. R. Philips (NYU School of Medicine, New York). The DsRed-Mito expression vector was from Clontech.

Cell Culture and Transfections—Rat-1 and Panc-1-derived cell lines were grown at 37 °C, 5% CO$_2$, in Dulbecco’s modified Eagle’s medium containing 4 mM glutamine, 10% fetal calf serum and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin), all from Biological Industries Beit Haemek. Parental cell lines (Rat-1, Panc-1) were from the American Type Culture Collection. Rat-1 cell lines stably expressing GFP-K-Ras(G12V) or GFP-H-Ras(G12V) (11) and the Panc-1 cell line expressing GFP-K-Ras(G12V) (35) were previously described. To generate pEGFP-HA-Rac1(G12V) or wild-type (WT) pEGFP-HA-H-Ras(WT), human HA-Rac1(G12V) (or human HA-H-Ras) were inserted into the PstI/BamHI sites of pEGFP-C3 (Clontech); Panc-1 cells stably expressing this construct were generated as described (35). Parental Panc-1 cells grown on glass coverslips were transiently transfected with YFP-K-Ras constructs (1 µg DNA) using FuGENE 6 (Roche Applied Science).

Drug Treatment—CPZ was dissolved in phosphate-buffered saline to 25 mM, and diluted (see figure legends) in cell growth medium containing 20 mM Hepes, pH 7.2. Water-dissolved W13 (2.86 mM) was diluted similarly.

Cell Fractionation and Immunoblotting—Panc-1 or Rat-1 cells grown in 10-cm dishes were treated (or not) with 25 µM CPZ or 25 µM W13 for 4 h (or 1 h), and subjected to hypotonic lysis followed by ultra centrifugation (100,000 × g, 30 min, 4 °C) to separate particulate (P) and soluble (S) fractions as described previously (36). 50 µg of protein (P fraction) and an equal percentage (v/v) of the S fraction were analyzed by SDS-PAGE and immunoblotting as described (37). Blots were probed with primary antibodies (see figure legends) followed by peroxidase-coupled secondary IgG (1:5,000, 1 h, 22 °C). Bands were visualized by enhanced chemiluminescence (ECL; Amersham Biosciences) and quantified by densitometry (Quantity One, Bio-Rad).

FRAP—Untreated or CPZ/W13-treated cells expressing a GFP-tagged Ras protein (see legend) were subjected to FRAP studies (37 °C, in Hepes-buffered medium, pH 7.2). An argon ion laser beam (Innova 70C, Coherent) was focused through a fluorescence microscope (Axio Imager.D1, Carl Zeiss MicroImaging) to a Gaussian spot of 0.77 ± 0.03 µm (×63 oil-immersion objective) or 1.17 ± 0.05 µm (×40 water-immersion objective), and experiments were conducted with each beam size (beam size analysis; as described in Refs. 38 and 39). The ratio between the illuminated areas was 2.28 ± 0.17 (n = 59). After a brief measurement at monitoring intensity (488 nm, 1 microwatt), a 5-milliwatt pulse (5–10 ms) bleached 60–75% of the fluorescence in the spot, and recovery was followed by the monitoring beam. The apparent characteristic fluorescence recovery time, τ, and the mobile fraction ($R_m$) were extracted from the FRAP curves by nonlinear regression analysis, fitting to a lateral diffusion process (38).

Methylene Blue Assay—24 h post-plating (5000 cells/well, 96-well plates), cells were treated with CPZ (see legends), fixed with 4% formaldehyde (2 h, 22 °C), and stained with 0.5% methylene blue (15 min, 22 °C). After elution with 0.1 M HCl, absorbance (OD at 595 nm) was measured with a Spectra Fluor (Tecan) microplate reader (40).

Cell Cycle Analysis by Flow Cytometry—Cells grown in 10-cm dishes and treated (or not) with CPZ or W13 were harvested by trypsinization, washed twice with phosphate-buffered saline, and resuspended in 1 ml of phosphate-buffered saline containing 0.05% Triton X-100 and 50 µg/ml propidium iodide. Samples were analyzed by fluorescence-activated cell sorter (FACS) flow cytometry (FACSort, BD Bioscience) using CellQuest Pro™ software.

Data Acquisition for Live Cell Imaging—Images were acquired with a motorized spinning disk confocal (Yokogawa CSU-22 Confocal Head) microscope (Axiovert 200M, Carl Zeiss MicroImaging) under control of SlideBook™ (Intelligent Imaging Innovations). Images were acquired with a ×63 oil
immersion objective (Plan Apochromat, NA 1.4). Three-dimensional image stacks were generated by sequential recordings along the z axis by varying the position of the piezoelectric-controlled stage. A step size of 0.4 μm was used for single time point acquisitions. Time-lapse series were acquired with 30-s intervals. For GFP and YFP excitation, samples were illuminated with a 40-milliwatt solid state 473 nm laser. For DsRed and Alexa 546 excitation, samples were illuminated with a 10-milliwatt solid state 561-nm laser. Typical exposure times were 0.5–1 s. 15–20 cells were imaged for each condition (untreated or treated with 25 μM CPZ, 1 h, 37 °C in HEPES-buffered medium with 10% fetal calf serum). Only cells that did not show gross morphological aberrations were subjected to quantification.

**Data Processing for Live Cell Imaging**—Three-dimensional images were either analyzed without further manipulations or were restored by the NearestNeighbours deconvolution or by the Constrained Iterative (CI) deconvolution (41) algorithms of SlideBook™. Fluorescence intensities of GFP- or YFP-Ras proteins were either the entirety of the cell or the cell interior were measured both in deconvoluted (supplemental Fig. S1B) or non-deconvoluted (supplemental Fig. S1A) images by a three step procedure: (i) definition of a region of interest (ROI) through intensity-based segmentation, leading to recognition of the entire GFP/YFP-Ras population of the cell (supplemental Fig. S1C); (ii) replication of this ROI and “frame by frame” deselection of regions identified as the cell interior; (iii) subtraction of the second ROI from the first, generating a region of interest spanning the entire interior of the cell (supplemental Fig. S1D). Internal GFP/YFP-Ras was calculated as the ratio between the fluorescence intensities of the cell interior ROI divided by the total cell ROI. 15–20 cells were measured for each condition. To ensure maximal clarity and consistency of presentation and quantification, both the images and bar graph of Fig. 2 depict images deconvoluted by the NearestNeighbours algorithm (Slidebook).

**Apoptosis Assays**—Apoptosis was measured on cells treated (or not) with CPZ (see legend to Fig. 4) by two independent methods. In the first, negatively charged phospholipids appearing at the outer cell surface early in the apoptotic process were labeled with Annexin V-biotin (1 μg/ml, 4 °C, 45 min) followed by Cy3-streptavidin (0.5 μg/ml, 4 °C, 45 min) in HEPES-buffered Hank’s balanced salt solution/HEPES supplemented with 2% bovine serum albumin. After fixation (4% paraformaldehyde, 45 min, 22 °C), fluorescent images were acquired with a spot RT CCD camera (Diagnostic Instruments) coupled to an Olympus IX70 microscope using a ×40 air objective. The second method was based on detection of caspase 3 activation. Cells grown in 6-well plates were treated (or not) with CPZ (see legend to Fig. 4). They were lysed in RIPA buffer as described (42), and subjected to SDS-PAGE and immunoblotting (37). Blots were probed with anti-active caspase 3 (1:500, 12 h, 4 °C) and peroxidase goat anti-mouse IgG (1:5000, 1 h, 22 °C). The bands were visualized by ECL and quantified as described above. Blots were acid-stripped (37) and reprobed with mouse anti-actin (1:1,000).

**RESULTS**

**CPZ Dislodges K-Ras but Not H-Ras from the Plasma Membrane**—At neutral pH, CPZ is an amphiphilic cation, whose association with the internal plasma membrane leaflet may counter negatively charged phospholipids. Because the membrane association of K-Ras partially depends on electrostatic interactions, we employed a Rat-1 cell line stably expressing GFP-K-Ras(G12V) (43) to investigate the effects of CPZ on the distribution of K-Ras(G12V) between cytosolic and membrane fractions. Cellular fractionation studies (Fig. 1, A and D) show that CPZ (25 μM) induced a significant (2-fold) reduction
Modulation of K-Ras by Chlorpromazine

![Graph showing the association of GFP-K-Ras(G12V) in Rat-1 cells. As shown in another calmodulin inhibitor (W13) affects the membrane CPZ is also a calmodulin inhibitor (31), we tested whether GFP-H-Ras(G12V) (previously characterized in Ref. 11) does not affect the membrane association of GFP-H-Ras(G12V) (Fig. 1, A). Only untreated, A, typical micrographs of three-dimensional images of untreated or CPZ-treated cells. The treated samples were maintained in a humidified incubator (5% CO2) until imaging. Only cells showing adequate morphology were imaged. Image acquisition was carried out over the entire cell volume (a z stack step size of 0.4 μm). Images were deconvoluted and quantified for GFP- or YFP-Ras fluorescence intensity in the different cellular compartments employing Slidebook software (see “Experimental Procedures”). Panels in A depict typical cells under the different conditions employed. The central square of each panel depicts one confocal mid-plane of the z stacks, whereas the upper and lateral smaller rectangles depict projections of x and yz dimensions, respectively. Panel B compiles the quantification (mean ± S.E.) of the increase in intracellular fluorescence in 15–20 cells per condition. CPZ (25 μM, 1 h, 37 °C) induced a significant increase (p < 0.05, Student’s t test) in the percentage of intracellular GFP-K-Ras(G12V), GFP-Rac1(G12V), and YFP-K-Ras(G12V,S171A,S181A,T183A); the latter lacks all phosphorylation sites in the hypervariable region. Similar results were observed in PANC-1 cells transfected with YFP-K-Ras(G12V,S181A) treated with CPZ (data not shown). A phosphomimetic mutation of Ser181 in K-Ras (the YFP-K-Ras(G12V,S181E) mutant) also resulted in a significant increase in intracellular localization as compared with GFP-K-Ras(G12V) (p < 0.05). In contrast, CPZ treatment (25 μM, 1 h, 37 °C) of PANC-1 cells stably expressing GFP-H-Ras did not induce a significant change in H-Ras intracellular distribution (p > 0.4). Bars, 10 μm.

of GFP-K-Ras(G12V) in the particulate (P) membrane fraction, accompanied by an increase in the cytoplasmic (S) fraction. CPZ also effectively reduced the membrane fraction of endogenous Ras in Rat-1 GFP-K-Ras(G12V) cells or in parental Rat-1 cells (52 ± 0.07% reduction in P; Fig. 1A), albeit with no detectable increase in the cytosolic fraction, possibly due to limited assay sensitivity.

To test whether the interference by CPZ with the membrane interactions of K-Ras is specific to this Ras isoform, we employed cellular fractionation studies on Rat-1 cells stably expressing GFP-H-Ras(G12V) (previously characterized in Ref. 11): no effect was observed on the membrane association of GFP-H-Ras(G12V) (Fig. 1, B and D). Furthermore, because CPZ is also a calmodulin inhibitor (31), we tested whether another calmodulin inhibitor (W13) affects the membrane association of GFP-K-Ras(G12V) in Rat-1 cells. As shown in Fig. 1 (B and D), W13 (25 μM) did not induce a significant reduction in the membrane-associated fraction of GFP-K-Ras(G12V), suggesting that if calmodulin inhibition is involved in the effect elicited by CPZ, its effect is relatively minor.

Activating mutations in K-Ras are often important in pancreatic tumors, and the tumorigenic phenotype of PANC-1 cells (a human pancreatic tumor cell line that naturally harbors constitutively active K-Ras) depends on the expression level of oncogenic K-Ras (35, 44, 45). We therefore examined the effects of CPZ on the membrane association of GFP-K-Ras(G12V) in a PANC-1-derived cell line that stably expresses this protein (35). In these cells, GFP-K-Ras(G12V) was mainly in the membrane fraction, but the relatively crude fractionation experiments were not sensitive enough to detect CPZ-mediated alterations in its membrane association (data not shown). However, this does not necessarily imply that CPZ fails to dislodge K-Ras from the plasma membrane in PANC-1 cells, because recent studies have shown that depending on the cellular and the biological context, various treatments can shift activated K-Ras from the plasma membrane to intracellular membrane compartments (13, 19, 21, 46, 47). This could leave GFP-K-Ras(G12V) in the membrane fraction even if it was dislodged from the plasma membrane. To explore this possibility, we employed immunofluorescent live confocal microscopy (Fig. 2). PANC-1 GFP-K-Ras(G12V) cells were left untreated or incubated with 25 μM CPZ for 1 h (37 °C). Typical z stacks (narrow rectangles on top and left) and median planes (central square panels) of cells before and after CPZ treatment are depicted in Fig. 2A, along with a quantification (derived from multiple independent measurements) of the relative increase in intracellular localized GFP-K-Ras(G12V) (Fig. 2). CPZ shifted GFP-K-Ras(G12V) from the plasma membrane to intracellular compartments (~90% increase; Fig. 2).

To examine whether the association with the plasma membrane of other GTPases harboring lysines in their membrane targeting region is also sensitive to CPZ, we examined the effect of the drug on the cellular distribution of GFP-Rac1(G12V) stably expressed in PANC-1 cells. Indeed, Fig. 2 (A and B) demonstrates a CPZ-mediated shift of GFP-Rac1(G12V) from the plasma membrane to the cell interior. It was recently shown that reducing the overall electrostatic charge at the C terminus of K-Ras by protein kinase C-mediated phosphorylation of Ser181 leads to its relocalization to endomembranes (13). Indeed, the phosphomimetic mutant YFP-K-Ras(G12V,S181E) showed significant intracellular localization in PANC-1 cells even without CPZ (Fig. 2). To test whether the CPZ effect on K-Ras(G12V) depends on phosphorylation of Ser/Thr in the K-Ras hypervariable region (Ser71, Ser181, and
Thi\textsuperscript{183}, see Ref. 13), we investigated its effect (25 \( \mu M \), 1 h) on the cellular distribution of GFP-K-Ras(G12V,S171A,S181A, T183A) in PANC-1 cells (Fig. 2, A and B). Although this mutant is non-phosphorylatable at these residues, it was effectively relocated intracellularly by CPZ. This supports the notion that phosphorylation in the K-Ras hypervariable region is dispensable for the CPZ-mediated effect. In accord with the fractionation studies in Rat-1 fibroblasts (Fig. 1, B and D), the cellular distribution of GFP-H-Ras(WT) in PANC-1 cells was not markedly altered by the CPZ treatment (Fig. 2, A and B); GFP- H-Ras(G12V) could not be measured in PANC-1 cells, due to the deleterious effects of its combined expression with the endogenous constitutively active K-Ras in these cells on their morphology.

**CPZ Enhances the Membrane to Cytoplasm Exchange of GFP-K-Ras(G12V)—**To directly characterize the effects of CPZ on the dynamic membrane interactions of GFP-K-Ras(G12V) or GFP-H-Ras (WT or the G12V mutant) in live PANC-1-cells, we conducted FRAP studies using two different laser beam sizes (beam size analysis) on untreated and on CPZ-treated cells (Fig. 3). FRAP beam size analysis characterizes the membrane interactions of proteins located to the inner leaflet of the plasma membrane, where the recovery of the FRAP signal can occur not only by lateral diffusion but also by exchange between membrane-bound and cytoplasmic pools. If FRAP occurs by lateral diffusion, \( \tau \) (for recovery) is the characteristic diffusion time \( \tau_{D} \), proportional to the illuminated area (\( \tau_{D} = \omega^{2}/4D \), where \( \omega \) is the Gaussian radius of the beam and \( D \) the lateral diffusion coefficient) (38)). When FRAP takes place by exchange, \( \tau \) reflects the chemical relaxation time, which is independent of the beam size (38). The \( \tau_{(X40)}/\tau_{(X63)} \) ratio expected for the two beam sizes generated using \( \times 40 \) and \( \times 63 \) objectives was 2.28 (the measured ratio between the illuminated areas) for pure lateral diffusion, \textit{versus} 1 for recovery by exchange. Intermediate values indicate a composite recovery mode, with a larger contribution of the faster process (38). In untreated PANC-1 GFP-K-Ras(G12V) cells, FRAP beam size analysis at 37 °C yielded \( \tau_{(X40)}/\tau_{(X63)} = 2.28 \), identical to the measured beam size ratio (Fig. 3D). Thus, FRAP of GFP-K-Ras(G12V) under these conditions occurs mainly by lateral diffusion, enabling to calculate \( D = 0.39 \mu m^{2}/s \). This value is in the same range reported earlier for various Ras proteins in different cell types (11, 43, 48). CPZ (25 \( \mu M \), 1 h, 37 °C) significantly reduced the

\[ \frac{\tau_{(X40)}}{\tau_{(X63)}} \]

ratio of GFP-K-Ras(G12V) to 1.7, suggesting a shift to FRAP by a mixture of lateral diffusion and exchange (Fig. 3D). Increasing CPZ to 50 \( \mu M \) (15–30 min) had a similar effect on the \( \tau \) ratio, and in addition reduced the \( \tau \) values, suggesting faster recovery kinetics (Fig. 3, C and D). In accord with the lack of effect on the distribution of GFP-H-Ras(G12V) observed by confocal microscopy (Fig. 2), CPZ had no significant effects on the \( \tau \) values or the \( \tau_{(X40)}/\tau_{(X63)} \) ratio of GFP-H-Ras(WT) (Fig. 3). Similar results (data not shown) were obtained with GFP-H-Ras(G12V) transiently expressed in PANC-1 cells. Treatment with W13 (25 \( \mu M \)), which had no significant effect on the membrane-associated fraction of GFP-K-Ras(G12V) in fractionation studies on Rat-1 cells (Fig. 1, B and D), was also ineffective in altering the mode of K-Ras membrane association (Fig. 3D); however, it induced a small increase in the FRAP times of K-Ras(G12V) at both beam sizes, indicating some perturbation in membrane organization (Fig. 3C). Increasing W13 to 50 \( \mu M \) reduced \( \tau_{(X63)} \) and \( \tau_{(X40)} \) to a similar extent, retaining the \( \tau_{(X40)}/\tau_{(X63)} \) value at 2.28 (pure lateral diffusion). Thus, a higher concentration of W13 increases the lateral diffusion rate of GFP-K-Ras(G12V), but does not significantly enhance its exchange rate. Similar experiments on Rat-1 GFP-K-Ras(G12V) cells yielded results in the same direction, exhibiting a reduction in the \( \tau_{(X40)}/\tau_{(X63)} \) ratio of GFP-
Modulation of K-Ras by Chlorpromazine

K-Ras(G12V) by 13% following CPZ treatment (25 μM), but no effect following incubation with 25 μM W13.

Taken together, the FRAP experiments imply that CPZ specifically affects the membrane interaction dynamics of GFP-K-Ras(G12V), resulting in increased dissociation from the plasma membrane, and a higher contribution of exchange. These effects correlate with the reduced association of GFP-K-Ras(G12V) with the plasma membrane (Figs. 1 and 2).
CPZ-mediated Dislodgment of GFP-K-Ras(G12V) from the Plasma Membrane Correlates with Apoptosis or Cell Cycle Arrest—The membrane association of Ras proteins is essential for their signaling and transforming activities (17). Furthermore, the cellular outcome of signaling by specific Ras proteins may depend on their targeting to the plasma membrane or to specific intracellular compartments (7, 8). Therefore, we examined whether the CPZ effects on the membrane localization of GFP-K-Ras(G12V) correlate with effects on cell growth and survival. Incubation with CPZ, for 24 h, at concentrations similar to those that dislodged GFP-K-Ras(G12V) from the membrane fraction, reduced the number of Rat-1 GFP-K-Ras(G12V) cells (Fig. 4, A and B). This effect, which correlates with the induction of cell death (see Fig. 4, C and D) was specific for K-Ras(G12V)-expressing Rat-1 cells, whereas the parental Rat-1 cells or Rat-1 GFP-H-Ras(G12V) cells responded in growth inhibition without reduction in cell numbers (panels A and B) and no apoptosis (panels C and D). To examine if the CPZ-mediated cell death in Rat-1 GFP-K-Ras(G12V) cells is apoptotic, we employed two independent approaches. First, treated and untreated live cells were stained with annexin V. Only Rat-1 GFP-K-Ras(G12V) cells treated with CPZ showed annexin V staining, indicative of the appearance of phosphatidylserine at the outer leaflet of the plasma membrane, characteristic of early stages of apoptosis (Fig. 4C). Second, we probed the ability of CPZ to activate caspase 3 in the different Rat-1 cell lines by immunoblotting. A marked enhancement in active caspase 3 was observed only in Rat-1 GFP-K-Ras(G12V) cells (Fig. 4D). Recently, translocation of oncogenic K-Ras to mitochondria was shown to correlate with the induction of apoptosis (13). To examine whether CPZ elicits similar effects, we subjected Rat-1 GFP-K-Ras(G12V) cells expressing DsRed-Mito to live cell confocal microscopy. In accord with its apoptosis-inducing effect (Fig. 4, C and D), CPZ (25 μM, 2 h, 37°C) led to a partial but reproducible relocalization of GFP-K-Ras(G12V) to mitochondria (Fig. 4E, arrows).

Interestingly, the effects of CPZ on cell fate depended on the cell type. In analogous studies on PANC-1 GFP-K-Ras(G12V) cells, CPZ (up to 50 μM) effectively inhibited cell growth, but unlike in Rat-1 GFP-K-Ras(G12V) cells, no cytotoxic effects (cell death) were observed (Fig. 5, A and B). At all CPZ concentrations tested, no staining with annexin V was detected (data not shown). FACS-based cell cycle analysis of the DNA content of PANC-1 GFP-K-Ras(G12V) cells (Fig. 5C) showed that CPZ reduces the percentage of cells in G2/M (2-fold), with a concomitant increase in the percentage of cells in G1/S. Importantly, CPZ treatment did not increase the percentage of cells in sub-G1, in accord with a lack of pro-apoptotic effect in these cells. Accordingly, even in PANC-1 cells showing a marked shift of GFP-K-Ras(G12V) to intracellular stores upon CPZ treatment (25 μM, 1 h, or 50 μM, 30 min), no localization in mitochondria was detected (Fig. 6). The conditions in these experiments did not arrest clathrin-mediated endocytosis of transferrin and resulted in partial colocalization of GFP-K-Ras(G12V) with Alexa 546-transferrin in endosomal compartments (Fig. 6, arrows). These results are in accord with previous reports (20) on translocation of K-Ras from the plasma membrane to early endosomes upon disruption of the electrostatic properties of the plasma membrane.

The motility of tumor cells and their ability to grow without anchorage correlate with their transformation state and invasive potential. To test whether CPZ affects the motility of PANC-1 GFP-K-Ras(G12V) cells, we performed a wound-healing scratch assay (Fig. 7). CPZ strongly inhibited in a dose-dependent manner the ability of these cells to close a wound inflicted to the monolayer. The CPZ-induced abrogation of the transformation of PANC-1 cells expressing K-Ras(G12V) was further confirmed by the inability of CPZ-treated cells to form colonies in soft agar (Fig. 7).

**DISCUSSION**

In the current studies, we combined biophysical and biochemical experiments to characterize the effects of the amphiphilic cationic drug, CPZ, on the interactions of constitutively active GFP-K-Ras(G12V) with the plasma membrane. Our findings show that CPZ specifically increases both the dynamics and extent of K-Ras(G12V) dissociation from the plasma membrane, and suggest that this effect is due to interference with electrostatic interactions of the K-Ras polybasic cluster with the membrane. Depending on the cellular context, these effects correlate with the ability of CPZ to inhibit cell growth or promote cell death. We propose a mechanism for the modulation of K-Ras membrane association and signaling by CPZ.

A high fraction of GFP-K-Ras(G12V) is membrane associated, although at least in Rat-1 cells a cytosolic fraction is also readily detected (Fig. 1). The steady-state association of GFP-K-Ras(G12V) with the plasma membrane was significantly weakened by CPZ both in Rat-1 fibroblasts and in PANC-1 cells, but the destination of GFP-K-Ras(G12V) molecules dislodged from the plasma membrane differed between the two cell types (Figs. 1, 2, 4, and 6). Thus, in Rat-1 cells, significant

---

**FIGURE 4. CPZ Induces apoptosis in GFP-K-Ras(G12V)-transformed Rat-1 cells.** Rat-1 cells (untransfected, stably expressing GFP-K-Ras(G12V) or stably expressing GFP-H-Ras(G12V)) were treated with 0, 10, or 25 μM CPZ for 24 (A and B) or 2 h (C–E). A, typical phase-contrast micrographs at 24 h, showing specific CPZ-induced cytotoxicity of Rat-1 GFP-K-Ras(G12V) cells. Bars, 100 μm. B, cell density assay by methylene blue. Data are presented as mean ± S.E. (n = 6) of the fold increase in absorbance (OD at 595 nm) relative to the value at the time of drug addition (zero time point). C, annexin V staining apoptosis assay. Parental Rat-1 cells (top panel group) were visualized by phase contrast; they did not show extracellular annexin V staining either before or after CPZ treatment. The Rat-1 GFP-K-Ras(G12V) cell line (mid-panel group), visualized by the GFP fluorescence present in all the cells, were markedly labeled by annexin V following incubation with CPZ, whereas Rat-1 GFP-H-Ras(G12V) cells (bottom panel group) were not. Bars, 20 μm. D, representative immunoblots of active caspase 3 (17 kDa) in Rat-1 cell lines with or without CPZ treatment. In Rat-1 GFP-K-Ras(G12V) cells, CPZ significantly elevated the level of active caspase 3 (67% increase ± 15%, p < 0.01; n = 3) but had no significant effect (p > 0.15) on active caspase 3 in Rat-1 or Rat-1 GFP-H-Ras(G12V) cells (0% increase ± 4 and 10% decrease ± 5%, respectively; n = 3). E, three-dimensional imaging of GFP-K-Ras(G12V) and DsRed-Mito in Rat-1 cells. Rat-1 GFP-K-Ras(G12V) cells plated on glass coverslips were transiently transfected with DsRed-Mito. After 24 h, untreated (upper panels) or CPZ-treated cells (lower panels) were subjected to three-dimensional imaging (0.4 μm z-stack step size) as in Fig. 2A, alternating between GFP and DsRed visualization. Arrowheads point at mitochondrial labeling (DsRed-Mito), whereas full arrows in the merged images indicate spots showing colocalization of GFP-K-Ras(G12V) with DsRed-Mito; CPZ induced modest but distinct colocalization of GFP-K-Ras(G12V) and the mitochondrial marker. Bars, 10 μm.
Modulation of K-Ras by Chlorpromazine

FIGURE 5. CPZ induces growth inhibition and G2/M cell cycle arrest in PANC-1 cells stably expressing GFP-K-Ras(G12V). A, representative phase-contrast micrographs of PANC-1 GFP-K-Ras(G12V) cells before (upper row) or after CPZ treatment (0, 10, or 25 μM; 24 h; lower row). Bars, 100 μm. B, cell density assay by methylene blue. PANC-1 cells stably expressing GFP-K-Ras(G12V) were treated with CPZ (0, 10, 25, or 50 μM) for 24 or 48 h, and subjected to the methylene blue assay. Data are presented as mean ± S.E. (n = 6) of the fold change in absorbance (OD at 595 nm) relative to the value at the time of drug addition (zero time point). At all concentrations tested, CPZ prevented the increase in absorbance (OD at 595 nm) relative to the value at the time of drug addition. Note that at both concentrations CPZ induced a significant (*, p < 0.02; **, p < 0.002) decrease (−2 fold) in the G0/G1 phase with a concomitant increase of the percentage of cells in the G2/M phase.

FIGURE 6. Three-dimensional imaging of GFP-K-Ras(G12V) with DsRed-Mito or Alexa 546-transferrin in PANC-1 cells. PANC-1 GFP-K-Ras(G12V) cells plated on glass coverslips were transfected with DsRed-Mito (upper row) or empty vector (lower row). After 24 h, they were incubated with 25 μM CPZ (1 h, 37 °C), either alone (upper row) or together with Alexa 546-transferrin (20 μg/ml). The cells were subjected to three-dimensional imaging of green (GFP) and red (DsRed-Mito or Alexa 546-transferrin) fluorescence as described in the legend to Fig. 4E. Bar, 10 μm. Unlike in Rat-1 cells, GFP-K-Ras(G12V) did not show detectable colocalization with DsRed-Mito in the CPZ-treated PANC-1 cells, but did show partial colocalization with the Alexa 546-transferrin endosomal marker. Arrowheads point at distinct intracellular vesicular staining of Alexa 546-transferrin; arrows indicate colocalized GFP-K-Ras(G12V). Bars, 10 μm.

fractionally, CPZ also interfered with the dynamics of the interactions of GFP-K-Ras(G12V) with the plasma membrane, as measured directly by FRAP beam size analysis (Fig. 3). Our FRAP results demonstrated that in PANC-1 cells CPZ altered the mode of recovery of GFP-K-Ras(G12V) from essentially pure lateral diffusion (negligible contribution of exchange) to a mixed contribution by lateral diffusion and exchange (τ(π×40)/τ(π×63) ratio, the ratio between the two laser beam sizes, shifted from 2.28 to 1.7). Because the laser beam in the FRAP experiments is focused on the plasma membrane in a confocal setup, this suggests that the exchange rate of GFP-K-Ras(G12V) between the plasma membrane and the cytoplasm increases significantly. Such a mixed mode of recovery has been previously observed for the association of GFP-tagged phospholipase Cβ2 with the inner plasma membrane leaflet (39).

Unlike GFP-K-Ras(G12V), GFP-H-Ras (WT or the G12V mutant) was largely insensitive to the CPZ effect (Figs. 1–3), reflecting the contribution of electrostatic interactions of the polybasic region (unique to K-Ras among the Ras isoforms) to its membrane association (17, 19, 20, 46). The concept that the CPZ-mediated dislodgment of GFP-K-Ras(G12V) from the plasma membrane is due to the partial neutralization of negatively charged phospholipids in the inner plasma membrane leaflet by the drug is supported by the sensitivity of GFP-Rac1(G12V), whose membrane association also depends on electrostatic interactions of a polybasic cluster, to dislodgment from the plasma membrane by CPZ (Fig. 2). This notion gains further support from several independent observations. (a) Tri-fluoperazine, a phenothiazine closely related to CPZ, was shown to bind to phospholipid membranes and to reduce their negative ζ potential (49). (b) CPZ was found to bind with higher affinity to biological membranes containing PI(4,5)P2 (50), which is enriched in the inner leaflet of the plasma membrane. (c) Proteins with polybasic clusters, including K-Ras and Rac1, were shown to dissociate from the plasma membrane upon
enzymatic depletion of PI(4,5)P₂ and PI(3,4,5)P₃ (19) or upon alteration of the inner surface potential during phagocytosis (21). (d) Reduction of the number of lysines in the K-Ras polybasic cluster reduced its association with the plasma membrane (46). (e) Protein kinase C-mediated phosphorylation of Ser¹⁸¹ (adjacent to the K-Ras polybasic domain) or its phosphomimetic mutation (S¹⁸¹E) lowered its affinity to the plasma membrane (13). Interestingly, in the latter two cases, the partial reduction in the positive charge of the K-Ras polybasic cluster resulted in its relocalization to intracellular organelles (13, 46), resembling the CPZ-mediated effect on GFP-K-Ras(G12V) (Figs. 2, 4, and 6). Importantly, the observation that CPZ can effectively relocalize the YFP-K-Ras(G12V,S¹⁸¹A) and YFP-K-Ras(G12V,S¹⁸¹A,T¹⁸³A) mutants to internal mem-

branes in PANC-1 cells demonstrates that the phosphorylation of Ser¹⁸¹ (and potentially Ser¹⁷¹ or Thr¹⁸³) is not required for the CPZ-induced relocalization (Fig. 2).

It is interesting to compare the effects of CPZ on the plasma membrane association of K-Ras with those of S-trans,trans-farnesylthiosalicylic acid (FTS), another drug known to dislodge Ras proteins from the plasma membrane (23). Unlike CPZ, treatment of cells expressing GFP-K-Ras(G12V) with FTS did not affect the mode of recovery in FRAP experiments, reflecting relatively stable membrane association (negligible contribution of exchange) in the presence of the drug (43). Rather, FTS mediated a time-dependent biphasic effect on the lateral diffusion rate of GFP-K-Ras(G12V) (initial enhancement and subsequent decrease) without altering the ratio of the percent of wound closure after 16 h (relative to time 0). The different effects of the two drugs suggest that they interfere with different tethering signals of K-Ras-electrostatic interaction by the polybasic domain in the case of CPZ, and the farnesyl group in the case of FTS.

Aside from its ability to partially neutralize negatively charged phospholipids, CPZ is a calmodulin inhibitor (31). This raises the possibility that part of its effect on K-Ras association with the plasma membrane may be due to calmodulin inhibition. However, although Ca²⁺-activated calmodulin can bind K-Ras and modulate its signaling, it mediates translocation of K-Ras from the plasma membrane to endomembranes (47); thus, inhibition of calmodulin by CPZ is expected to counter K-Ras dissociation from the plasma membrane, contrary to the observed effects (Figs. 1–3). The notion that the CPZ-mediated dislodgment of GFP-K-Ras(G12V) from the plasma membrane is not due to inhibition of calmodulin is further supported by the failure of another calmodulin inhibitor, W13, to induce such effects (Figs. 1 and 3). Taken together, our findings suggest that the main mechanism by which CPZ interferes with the plasma membrane tethering of K-Ras is based on compromis-

ing the electrostatic interactions between the K-Ras polybasic cluster and negatively charged phospholipids in the inner leaf-

let of the plasma membrane.

We propose that following desorption from the plasma membrane, the intracellular destination of K-Ras(G12V) depends at least in part on the cellular context. Thus, in Rat-1 GFP-K-Ras(G12V) cells, CPZ treatment resulted in marked localization of GFP-K-Ras(G12V) in the cytoplasm and endo-

membranes, including mitochondria (Figs. 1 and 4E). In accord with a recent report that mitochondrial localization of K-Ras(G12V) initiates apoptosis (13), CPZ effectively mediated apoptosis of Rat-1 GFP-K-Ras(G12V) cells (Fig. 4). On the other hand, treatment of PANC-1 GFP-K-Ras(G12V) cells with CPZ did not lead to detectable localization in mitochondria, and GFP-K-Ras(G12V) was shifted to other internal organelles, including endosomes (Fig. 6). In accord with the lack of distinct CPZ-mediated relocalization of K-Ras(G12V) in these cells to mitochondria, CPZ did not initiate apoptosis of PANC-1 GFP-K-Ras(G12V) cells, but rather arrested them in the G₂/M phase of the cell cycle (Fig. 5). These findings are in accord with the demonstration that oncogenic Ras accelerates G₂/M transition and abrogates the G₂ DNA damage and mitotic spindle checkpoints (51). Furthermore, ablation of Ras downstream effectors was shown to arrest the cell cycle in the G₂/M phase (52). The

![Figure 7](image-url)
Modulation of K-Ras by Chlorpromazine

dependence of the biological outcome of the interference with K-Ras membrane interaction on the cellular context (Figs. 4–7) is in line with the cell type-dependent effects of small interfering RNA-mediated depletion of oncogenic K-Ras: whereas PANC-1 cells responded in cell cycle arrest, MIA-PACA cells underwent apoptotic cell death (45).

The interactions described above may have far-reaching biological consequences on cell fate. It has long been known that phenothiazines in general and CPZ in particular have growth-inhibitory effects on many tumor cell lines and some in vivo tumor models (31). However, the mechanism(s) of these effects were not determined. Therefore, the mechanism proposed here may be highly relevant for tumor cell lines or tumors that depend on overactivation of K-Ras. Indeed, CPZ strongly inhibited both the motility and the anchorage-independent growth in soft agar of human pancreatic carcinoma cells (PANC-1 GFP-K-Ras(G12V); Fig. 7), an effect that may also include a contribution of CPZ-mediated RalGDS displacement. The current results underscore the potential use of agents that perturb the interactions of specific oncogenes (e.g. constitutively active K-Ras) with their target membranes for anti-tumorigenic treatments.

Acknowledgments—We thank Dr. Mark R. Phillips for the YFP-tagged K-Ras constructs and Dr. Yoel Kloog for helpful comments.

REFERENCES
1. Hancock, J. F., and Parton, R. G. (2005) Biochem. J. 389, 1–11
2. Bos, J. L. (1989) Cancer Res. 49, 4682–4689
3. Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827
4. Voice, J. K., Klemke, R. L., Le, A., and Jackson, J. H. (1999) J. Biol. Chem. 274, 17164–17170
5. Walsh, A. B., and Bar-Sagi, D. (2001) J. Biol. Chem. 276, 15609–15615
6. Koera, K., Nakamura, K., Nakao, K., Miyoshi, J., Toyoshima, K., Hatta, T., Otani, H., Aiba, A., and Katsuki, M. (1997) Oncogene 15, 1151–1159
7. Kfir, S., Ehrlich, M., Goldshmid, A., Liu, X., Kloog, Y., and Henis, Y. I. (2003) Mol. Cell. Biol. 23, 8645–8652
8. Quatela, S. E., and Philips, M. R. (2006) Curr. Opin. Cell Biol. 18, 162–167
9. Prior, J. A., Muncke, C., Parton, R. G., and Hancock, J. F. (2003) J. Cell Biol. 160, 165–170
10. Niv, H., Gutman, O., Kloog, Y., and Henis, Y. I. (2002) J. Cell Biol. 157, 865–872
11. Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., 2nd, Cox, A. D., and Philips, M. R. (2002) Nat. Cell Biol. 4, 343–350
12. Bivona, T. G., Quatela, S. E., Bodemann, B. O., Ahearn, I. M., Soksis, M. J., Mor, A., Miura, J., Wiener, H. H., Wright, L., Saba, S. G., Yim, D., Fein, A., Perez de Castro, I., Li, C., Thompson, C. B., Cox, A. D., and Philips, M. R. (2006) Mol. Cell 21, 481–493
13. Eisenberg, S., Shvartsman, D. E., Ehrlich, M., and Henis, Y. I. (2006) Mol. Cell Biol. 26, 7190–7200
14. Laude, A. J., and Prior, I. A. (2008) J. Cell Sci. 121, 421–427
15. Zhang, F. L., and Casey, P. J. (1996) Annu. Rev. Biochem. 65, 241–269
16. Hancock, J. F., Paterson, H., and Marshall, C. J. (1990) Cell 63, 133–139
17. Hancock, J. F., Cadwallader, K., Paterson, H., and Marshall, C. J. (1991) EMBO J. 10, 4033–4039
18. Heo, W. D., Inoue, T., Park, W. S., Kim, M. L., Park, B. O., Wandless, T. J., and Meyer, T. (2006) Science 314, 1458–1461
19. Gomez, G. A., and Daniotti, L. J. (2007) FEBS J. 274, 2210–2228
20. Yeung, T., Terebiznik, M., Yu, L., Silvius, J., Abidi, W. M., Philips, M., Levine, T., Kapus, A., and Grinstein, S. (2006) Science 313, 347–351
21. Basso, A. D., Kirschmeyer, P., and Bishop, W. R. (2006) J. Lipid Res. 47, 15–31
22. Kloor, Y., and Cox, A. D. (2004) Semin. Cancer Biol. 14, 253–261
23. Friday, B. B., and Adjei, A. A. (2005) Biochem. Biophys. Acta 1756, 127–144
24. Lopez-Munoz, F., Alamo, C., Cuenca, E., Shen, W. W., Clervoy, P., and Rubio, G. (2005) Ann. Clin. Psychiatry 17, 113–135
25. Schrier, S. L., Zachowski, A., and Devaux, P. F. (1992) Blood 79, 782–786
26. Golebiewska, U., Gambhir, A., Hangyas-Mihalyne, G., Zaitseva, I., Radler, J., and McLaughlin, S. (2006) Biochem. J. 39, 588–599
27. McLaughlin, S., Wang, J., Gambhir, A., and Murray, D. (2002) Annu. Rev. Biophys. Biomol. Struct. 31, 151–175
28. Sofer, A., and Futerman, A. H. (1995) J. Biol. Chem. 270, 12117–12122
29. Ford, I. M., Prozialeck, W. C., and Hain, W. N. (1989) Mol. Pharmacol. 35, 105–115
30. Nordenberg, J., Fenig, E., Landau, M., Weizman, R., and Weizman, A. (1999) Biochem. Pharmacol. 58, 1229–1236
31. Motohashi, N., Kawase, M., Saito, S., and Sakagami, H. (2000) Curr. Drug Targets 1, 237–245
32. Pollack, A., and Levij, I. S. (1972) Cancer Res. 32, 1912–1915
33. Fujita, K., Iwase, S., Ito, T., and Matsumiya, M. (1958) Nature 181, 54
34. Dreissigacker, U., Mueller, M. S., Unger, M., Siegert, P., Genze, F., Gierisch, P., and Giehl, K. (2006) Cell Signal. 18, 1156–1168
35. Hakki, R., Gana-Weisz, M., Elad, G., Paz, A., Marciano, D., Egozi, Y., Ben-Baruch, G., and Kloog, Y. (1998) Biochemistry 37, 1306–1314
36. Fleming, J. B., Shen, G. L., Holloway, S. E., Davis, M., and Brekken, R. A. (2004) Mol. Cancer Res. 2, 7317–7322
37. Basso, A. D., Kirschmeyer, P., and Bishop, W. R. (2006) J. Cell Biol. 170, 481–493