CHAPTER SEVEN

Analysis of K-Ras Phosphorylation, Translocation, and Induction of Apoptosis

Steven E. Quatela, Pamela J. Sung, Ian M. Ahearn, Trever G. Bivona, *and* Mark R. Philips

Contents

Departments of Medicine, Cell Biology, and Pharmacology and the Cancer Institute, New York University School of Medicine, New York

Methods in Enzymology, Volume 439
ISSN 0076-6879, DOI: 10.1016/S0076-6879(07)00407-7

Abstract

K-Ras is a member of a family of proteins that associate with the plasma membrane by virtue of a lipid modification that inserts into the membrane and a polybasic region that associates with the anionic head groups of inner leaflet phospholipids. In the case of K-Ras, the lipid is a C-terminal farnesyl isoprenoid adjacent to a polylysine sequence. The affinity of K-Ras for the plasma membrane can be modulated by diminishing the net charge of the polybasic region. Among the ways this can be accomplished is phosphorylation by protein kinase C (PKC) of serine 181 within the polybasic region. Phosphorylation at this site regulates a farnesyl-electrostatic switch that controls association of K-Ras with the plasma membrane. Surprisingly, engagement of the farnesyl-electrostatic switch promotes apoptosis. This chapter describes methods for directly analyzing the phosphorylation status of K-Ras using metabolic labeling with ^{32}P, for indirectly assessing the farnesyl-electrostatic switch by following GFP-tagged K-Ras in live cells, for artificially activating the farnesyl-electrostatic switch by directing the kinase domain of a PKC to activated K-Ras using a Ras-binding domain, and for assessing apoptosis of individual cells using a YFP-tagged caspase 3 biosensor.

1. Introduction

The differential biology of Ras isoforms is generated, in large part, by distinct membrane targeting sequences. Membrane association of all Ras isoforms requires farnesylation, proteolysis, and carboxyl methylation of a C-terminal CAAX motif. Plasma membrane (PM) targeting of the principal splice variant of K-Ras also requires a unique polybasic region adjacent to the CAAX motif (Choy *et al.*, 1999; Hancock *et al.*, 1990; Jackson *et al.*, 1994). K-Ras thus falls into a broad class of proteins that are anchored to the cytoplasmic face of the PM by virtue of post-translational modification with lipids that act in conjunction with polybasic stretches of polypeptide. Whereas the lipid moieties are thought to insert into the phospholipid bilayer, the polybasic regions are believed to associate with the anionic head groups of inner leaflet phospholipids (Leventis and Silvius, 1998).

Included in this class of proteins is the myristoylated alanine-rich C kinase substrate (MARCKS) that associates with the PM via an N-terminal myristoyl modification and a polybasic region. Serine residues within the polybasic region are sites for phosphorylation, mediated by protein kinase C (PKC), that neutralize the charge and thereby cause the MARCKS protein to dissociate from the PM. The mechanism by which MARCKS is discharged from the PM through phosphorylation has been referred to as a myristoyl electrostatic switch (McLaughlin and Aderem, 1995).

Like MARCKS, the polybasic region of K-Ras harbors three potential phosphorylation sites, and this segment has been shown previously to be phosphorylated by PKC (Ballester *et al.*, 1987). We therefore tested the hypothesis that phosphorylation of the C-terminal segment of K-Ras might regulate its association with the PM and thereby constitute a farnesyl-electrostatic switch. Using green fluorescent protein (GFP)-tagged Ras proteins and live cell imaging, we have observed that PKC agonists induce a rapid translocation of K-Ras from the PM to intracellular membranes that include the endoplasmic reticulum (ER), Golgi apparatus, and, surprisingly, the outer mitochondrial membrane. Interestingly, phosphorylation and internalization of K-Ras also stimulated apoptosis. These observations show that the subcellular localization and function of K-Ras are modulated by PKC in a novel pathway resulting in cell death (Bivona *et al.*, 2006). This chapter describes assays that enabled us to determine which site(s) in the C-terminal domain of K-Ras is phosphorylated by PKC, to confirm that it is PKC phosphorylation of K-Ras that is responsible for its internalization, and to monitor the onset of apoptosis in individual cells in response to various K-Ras constructs.

2. Analysis of K-Ras Phosphorylation by Metabolic Labeling with ^{32}P

Previous studies performed by Ballester *et al.* (1987) demonstrated the presence of a PKC substrate in the C terminus of K-Ras. The C-terminal region of K-Ras harbors three potential phosphate acceptors: S171, S181, and T183. However, only serines conform to consensus phosphorylation sites, and phospho-amino acid analysis of K-Ras from cells exposed to PKC agonists revealed only phosphoserine (Ballester *et al.*, 1987). Sequence analysis can be used to determine the most likely site of phosphorylation based on the consensus PKC site, S/T-X-R/K. For K-Ras, S181 within the C-terminal membrane-anchoring region of K-Ras conforms most closely to a consensus PKC site.

Metabolic labeling of cells with ^{32}P is the standard method for monitoring the level of protein phosphorylation. Combining metabolic labeling with the expression of K-Ras constructs with mutated phosphate acceptor sites is a relatively easy way to determine the precise site of PKC phosphorylation of K-Ras or other phosphorylated proteins. One caveat is that it remains possible that mutating a phosphate acceptor site can have an indirect effect on a nearby site by altering the conformation of the region and lowering the efficiency of phosphorylation. Nevertheless, substitution of phosphate nonacceptors such as alanine for acceptors can give important information regarding protein phosphorylation. To do so, cells are transfected with expression vectors

encoding the protein of interest possessing nonphosphorylatable alanine residues at potential phosphate acceptor sites. After a suitable period to allow expression, the transfected cells are labeled metabolically with ^{32}P. The cells are then stimulated with a PKC agonist, such as bryostatin-1 or phorbol myristate acetate (PMA), to induce phosphorylation and are lysed in a RIPA buffer. The lysates are immunoprecipitated for the protein of interest, which is then subjected to SDS-PAGE, and radioactive phosphate incorporation is observed by PhosphorImaging.

2.1. Transfection and metabolic labeling

Twenty-four hours before transfection, COS-1 cells are seeded into six-well plates at a density of 2.0×10^5 cells per well. The cells should be grown in 5% CO_2 at 37° in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics to 70% confluence on the day of transfection. The wild-type and mutated K-Ras alleles cloned into a mammalian expression vector can be transfected into COS-1 cells with high efficiency using SuperFect (Qiagen). Lipofectamine (Invitrogen) used according to the manufacturer's instructions gives similar results. Three micrograms of each plasmid DNA is transfected with 7 μl SuperFect reagent in 100 μl unmodified DMEM. After 15 min, when transfection complexes have been allowed to form, 600 μl of DMEM supplemented with 10% FBS and antibiotics is added to bring the volume up to 700 μl. In the meantime, the medium from each well of the six-well plate is aspirated, and cells are washed with prewarmed phosphate-buffered saline (PBS). The entire transfection mixture is added to a single well, and cells are incubated for 3 h at 37° in 5% CO_2. The medium with the transfection complex is then removed, the cells are washed once with prewarmed PBS, and fresh DMEM containing serum and antibiotics is added to the cells. The cells are then grown for 24 h at 37° in 5% CO_2 to allow expression of the constructs.

Twenty-four hours after transfection, the COS-1 cells are ready for metabolic labeling. Cells should not have grown to more than 90% confluence, as phosphate uptake is maximal in rapidly growing cells. Growth medium containing serum and antibiotics is removed by aspiration. Cells are then washed three times with prewarmed phosphate-free DMEM to remove any residual phosphate-containing medium. Labeling medium is then prepared, containing 0.5 mCi/ml [^{32}P]orthophosphate (Perkin Elmer; NEM) in prewarmed phosphate-free DMEM supplemented with 10% partially dialyzed FBS. A final concentration of inorganic phosphate of 50 to 100 μM should be maintained to prevent slowing down of cellular metabolism. Serum dialyzed against phosphate-free saline can be mixed with undialyzed serum at a ratio of 1:3 to provide the required concentration of phosphate in the labeling medium. One milliliter of labeling media is

added to each well of the six-well plate. The plate is then placed in a warmed Plexiglas box (to shield β radiation), and the box is put in the incubator at 37° in 5% CO_2 for a labeling period of 6 h. This intermediate period of labeling strikes a balance, ensuring that enough time elapses so that an equilibrium between cellular and extracellular phosphate pools is reached and ATP pools are labeled to reasonably high specific activity on the one hand, while limiting the radiation damage to cells caused by longer periods of labeling on the other.

2.2. Stimulation, immunoprecipitation, SDS-PAGE, and analysis of phosphorylation of K-Ras

At the end of the labeling period, the six-well dish is removed from the Plexiglas box and the labeling medium is removed manually using a disposable Pasteur pipette. Liquid ^{32}P waste from this and subsequent steps must be handled with caution and disposed of appropriately. The cells are then washed once with prewarmed phosphate-free DMEM. Then prewarmed phosphate-free DMEM is added with or without a PKC agonist, such as bryostatin-1 (100 n*M*). The six-well dish is again placed in the Plexiglas box and placed in the incubator at 37° with 5% CO_2 for 20 min. After the stimulation period is finished, the dish is removed from the incubator and the medium is removed from all wells with a Pasteur pipette. The cells are now washed three times with ice-cold TBS, with the wash buffer removed each time manually as before. The cells are lysed directly in the six-well plate, using 1 ml RIPA buffer [20 m*M* Tris-HCl, pH 7.5, 150 m*M* NaCl, 1% NP-40, 0.1% SDS, 0.1% Na-deoxycholate (DOC), 0.5 m*M* EDTA, protease inhibitor tablet (Roche), 10 n*M* microcystin, 50 m*M* NaF, and 0.2 m*M* Na_2VO_3] per well. The plate is left at 4° for 20 min to allow complete lysis, after which time the lysates are scraped from the dish and transferred to microcentrifuge tubes. The lysates are then clarified to remove detergent-insoluble material by centrifuging at 13,000 rpm for 15 min. After centrifugation, the lysate supernatant is transferred to a new microcentrifuge tube and is ready for immunoprecipitation.

To the 1 ml of clarified lysates, 30 μl agarose (1:1 slurry of beads:buffer) conjugated with Y13–259 rat monoclonal anti-Ras antibody (Santa Cruz Biotechnologies) is added and the mixture is rotated at 4° for 2 h. Other phosphorylated proteins can be similarly immunoprecipitated using cognate antibodies and protein A or G agarose beads. After immunoprecipitation, the agarose beads are washed three times with RIPA lysis buffer (containing protease inhibitors, microcystin, NaF, and Na_2VO_3), pelleted by centrifugation at 7000 rpm for 3 min, and eluted by boiling in 30 μl of SDS sample buffer. Lysates from parallel plates not labeled metabolically can be used to check for expression of the constructs. Eluates are then loaded onto 14% Tris-glycine gels and subjected to electrophoresis, along with the nonlabeled lysates.

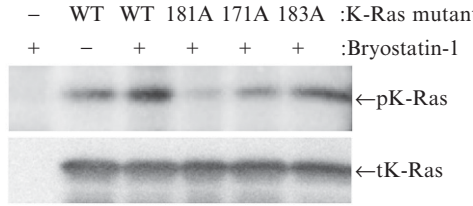

Figure 7.1 Mapping sites of K-Ras phosphorylation by metabolic labeling with ^{32}P. COS-1 cells were transfected with expression constructs for the indicated K-Ras alleles and 16 h later were labeled metabolically for 6 h with [^{32}P]orthophosphate. Ras was immunoprecipitated from cell lysates, and phosphoproteins were visualized by PhosphorImager. Cells treated in parallel that were not exposed to [^{32}P]orthophosphate were used for Ras immunoblots (bottom) to confirm equivalent expression.

After electrophoresis has progressed to the point where the proteins of interest can be resolved, the gel is removed from the chamber and dried on Whatman 3MM filter paper for 3 h at 70°. An identical gel is run with the immunoprecipitates of unlabeled cells, transferred to nitrocellulose, and immunoblotted with Ras10 pan-Ras monoclonal antibody (Calbiochem). The dried gel is then exposed to a PhosphorImager plate at room temperature and scanned by a laser after 10 h. Relative levels of radioactive phosphate incorporation into the various proteins are quantified by ImageQuant software.

Bryostatin-1 induced phosphorylation of K-Ras that was markedly diminished with an S181A substitution, confirming S181 as the major phosphate acceptor (Fig. 7.1) (Bivona *et al.*, 2006). However, the low level of ^{32}P incorporation into the 181A mutant suggests that, although S181 is the primary site, other minor sites of phosphorylation are likely. The diminished signal in the S171A mutant suggests that this residue might also serve as a phosphate acceptor. In contrast, a T183A substitution did not diminish ^{32}P incorporation, confirming that phosphorylation of this residue is not involved in the farnesyl-electrostatic switch of K-Ras.

3. Phoshorylation of Endogenous K-Ras in T Cells

The observation that overexpressed GFP-K-Ras in COS-1 cells responds to direct activation of PKC by diacylglycerol analogs suggested that this effect could also be observed in a more physiologic setting. To this end, we studied lymphocytes that are well known to activate both Ras (Downward *et al.*, 1990) and PKC (Valge *et al.*, 1988) following engagement of the T-cell receptor (TCR). Metabolic labeling of Jurkat T cells with ^{32}P followed by cross-linking of the TCR is therefore a good system

with which to study phosphorylation of endogenous K-Ras following a physiologically relevant stimulus.

3.1. Labeling of Jurkat cells

Metabolic labeling of Jurkat T cells involves slight modifications from the procedure described earlier, owing to the fact that they are nonadherent cells. Moreover, because one is interested in labeling endogenous proteins in these cells, no transfection is necessary. The Jurkat T-cell culture should be maintained at a concentration between 10^5 and 10^6 cells/ml of medium, and the cells should appear healthy and double regularly. Immediately before labeling, the Jurkat cells (4×10^7) are washed three times with prewarmed phosphate-free DMEM and finally resuspended in phosphate-free DMEM supplemented with 10% FBS (undialyzed:dialyzed, 3:1) to a concentration of 10^7 cells/ml of medium. Although RPMI is the standard medium for Jurkat T cells, it contains a high concentration of phosphate and therefore phosphate-free DMEM is used instead. [^{32}P]orthophosphate is then added to the labeling medium to a final concentration of 0.5 mCi/ml. The resuspended cells in their labeling media can then be transferred to a 60-mm dish, placed in a Plexiglas box, and put in an incubator at 37° in 5% CO_2 for a labeling period of 6 h.

3.2. Stimulation, immunoprecipitation, SDS-PAGE, and analysis of endogenous K-Ras phosphorylation by anti-CD3

After the labeling period, Jurkat cells are washed with prewarmed DMEM as described earlier for COS-1 cells. However, because Jurkat cells grow in suspension, they must be pelleted after each wash. After the final wash, the cells are resuspended in 2 ml (2.0×10^7 cells/ml) prewarmed DMEM and are divided equally into two microcentrifuge tubes. The cells in one tube are then stimulated with 10 μg/ml anti-CD3 antibodies (Ancell, Bayport MN) and the other with buffer control. The tubes are placed in a rack in the Plexiglas box and put in the incubator for 30 min at 37° with 5% CO_2. After stimulation, cells are washed three times with cold TBS. The cells are finally pelleted by centrifugation at 7000 rpm for 3 min, resuspended in 500 μl TBS, and lysed with an additional 500 μl of 2× RIPA buffer. The cells are lysed for 15 min, rotating at 4°, after which the lysates are clarified by centrifugation. Total Ras protein or other proteins of interest are immunoprecipitated as described previously. The immunoprecipitated protein is washed and eluted by boiling with SDS sample buffer, and eluates are loaded onto a 14% Tris-glycine gel and subjected to electrophoresis. The gel is then dried on Whatman 3MM filter paper and placed in a PhosphorImager plate for 3 to 6 h, and the plate is scanned in a PhosphorImager to visualize

radioactive phosphate incorporation. Using this method, phosphorylation of endogenous Ras can be observed in Jurkat T cells following stimulation of the TCR, demonstrating Ras phosphorylation in response to physiological signaling.

4. Analysis of K-Ras Farnesyl-Electrostatic Switch by Live Cell Imaging

Having defined the phosphate acceptor sites for PKC in the C-terminal membrane targeting region of K-Ras4B by metabolically labeling as described earlier, we now determine the effects of phosphorylation at those sites on K-Ras association with the plasma membrane. This is best accomplished with live cell imaging in which GFP-tagged Ras constructs are expressed in a well-behaved cell type and then localization is scored in real time before and at various times after the addition of a PKC agonist. To identify endomembrane compartments that serve as acceptors for GFP-K-Ras or YFP-K-Ras dislodged from the plasma membrane, one can use various compartment markers.

4.1. Cloning and transfection

GFP-tagged K-Ras12V constructs are cloned in two steps. First, the K-Ras12V coding region is polymerase chain reaction (PCR) amplified with in-frame 5′-*Hin*dIII and 3′-*Apa*I linkers designed into the primers. The amplification product is digested with *Hin*dIII and *Apa*I and is then ligated into the pEGFP-C1 vector (Clontech), which has been linearized by double digestion with *Hin*dIII and *Apa*I and purified. It is important to use an N-terminal GFP tag to allow for proper processing of the CAAX motif of K-Ras. Second, the S171, S181, or T183 residues can be mutated to alanine or glutamic acid using QuikChange XL (Stratagene) site-directed mutagenesis according to the manufacturer's instructions. Combinations of mutations (e.g., GFP-K-Ras12V171A183A) can be created by sequential mutagenesis of each codon.

Twenty-four hours before transfection, 2.0×10^5 MDCK cells are seeded in 35-mm culture dishes that incorporate a glass coverslip in the center (MatTek) in 2 ml DMEM, supplemented with 10% FBS and antibiotics. The cells should be grown to 70% confluence on the day of transfection. Two micrograms of each GFP-tagged K-Ras12V construct is combined with 7 μl SuperFect reagent in 100 μl unmodified DMEM. After 15 min to allow transfection complexes to form, 600 μl of DMEM, supplemented with 10% FBS and antibiotics, is added to bring the volume of the transfection mixture

up to 700 μl. In the meantime, the medium from each dish is aspirated, and cells are washed with prewarmed PBS. The entire transfection mixture is added to a single dish, and cells are allowed to incubate for 3 h at 37° in 5% CO_2. The medium with the transfection complex is then removed, cells are washed once with prewarmed PBS, and fresh DMEM containing serum and antibiotics is added to the cells.

4.2. Stimulation and visualization of translocation

Twenty-four hours after transfection, living cells are visualized with a Zeiss 510 inverted laser-scanning confocal microscope (LSM) using a high magnification, high NA objective (e.g., Plan-Neofluar 63x/1.25) and GFP or FITC narrow band filters. A microincubator (e.g., PDMI-2 from Harvard Apparatus) should be employed to keep the cells at 37°. A CO_2 microincubator (e.g., Incubator S-M from Zeiss) can be used, but is not necessary, as the cells will be studied for less than 30 min after removal from the incubator. Cells for imaging should be selected on the basis of morphology and intermediate fluorescent intensity. GFP-K-Ras12V in unstimulated cells should appear to localize exclusively at the PM. In a confluent monolayer of MDCK cells, PM localization should be easily discernible, as it appears as a ''chicken-wire'' pattern due to cell–cell contact. Whether imaging individual cells or a cell that is in a confluent patch of monolayer, one should choose a cell that appears healthy and has a structure decorated with GFP that can be unambiguously interpreted as plasma membrane. Sometimes it helps to acquire a shallow Z stack of three to five 0.45-μm slices to afford more opportunity to score plasma membrane and internal membranes. This is particularly important when the agents employed, such as bryostatin-1, can cause an acute shape change of the cell. The brightest of cells often have irregular morphology and fluorescent structures that may be artifacts and are therefore best avoided. The dimmest cells, although often of excellent morphology, may bleach below the threshold for high-quality imaging during stimulation and image acquisition such that these should also be avoided. The best cells to follow have intermediate fluorescence intensity.

Once an appropriate cell or group of cells is selected, they are stimulated while under continuous observation by adding a PKC agonist, such as 100 n*M* bryostatin-1 or 100 n*M* PMA with or without 500 ng/ml ionomycin, directly into the 35-mm MatTek plates. Thorough mixing is accomplished by pipetting 200 to 500 μl of the media two times with a 1000-μl pipette. Mixing must be done in an extremely gentle fashion so as not to lose the cell of interest from the field or focal plane. Scanned images (single or a shallow Z stack) should be acquired before and every 1 to 5 min after adding drug.

Following stimulation, GFP-K-Ras12V should translocate to internal membranes rapidly (Fig. 7.2) (<1 min onset, 3 min for maximal effect). The translocation event can also be observed with GFP-K-Ras12V171A183A, suggesting that phosphorylation of these residues is not required for the farnesyl-electrostatic switch. However, GFP-K-Ras12V181A does not translocate, and GFP-K-Ras12V with a glutamic acid at position 181 to mimic phosphorylation is internalized constitutively, confirming that serine 181 is the critical residue that controls the farnesyl-electrostatic switch. Thus, live cell imaging can confirm that serine 181 is necessary and sufficient for PKC-induced translocation (Bivona *et al.*, 2006). Because this is the same residue found to be the principal phosphate acceptor (see earlier discussion), one can deduce that phosphorylation at this residue regulates the farnesyl-electrostatic switch of K-Ras.

4.3. Colocalization of phosphorylated K-Ras with compartment markers

To determine the localization of K-Ras or other proteins of interest following translocation to intracellular compartments, one can study cells cotransfected with K-Ras tagged with a fluorescent protein (FP) and a compartment marker tagged with a different FP that can be spectrally resolved from FP-K-Ras. YFP-K-Ras is an attractive option that can be used in combination with compartment markers tagged with CFP or mCherry. To visualize organelles such as ER, Golgi, endosomes, and mitochondria, one should choose a relatively large and well-spread cell type

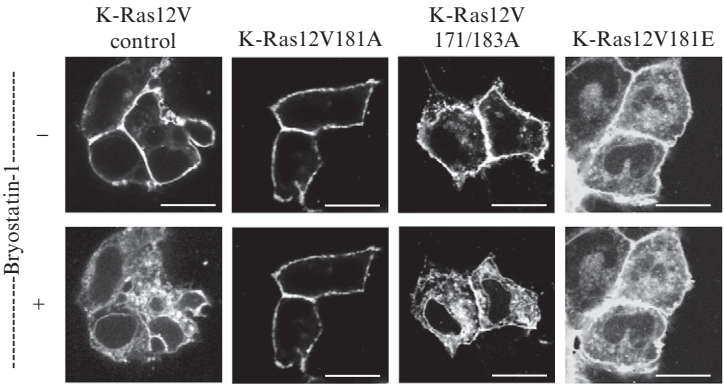

Figure 7.2 Translocation of GFP-K-Ras in response to PKC stimulation. MDCK cells were transfected with expression constructs for the indicated K-Ras alleles and 16 h later were imaged alive with a Zeiss 510 LSM before and 5 min after the addition of bryostatin-1. Constitutively active GFP-K-Ras12V translocates, as does GFP-K-Ras12V171/183A. In contrast, GFP-K-Ras12V181A remains on the plasma membrane and GFP-K-Ras12V181E is constitutively internalized. Bars indicate 10 μm.

such as COS-1. HEK293 and NIH/3T3 are not well suited for live cell imaging to determine subcellular localization, as the former are relatively small and poorly adherent and the latter are elongated. Epithelial cells such as MDCK or HeLa work well both for identifying intracellular organelles and for scoring for plasma membrane, as confluent cells grow with a semicolumnar geometry.

To mark the ER, we use the first transmembrane domain of the avian infectious bronchitis virus M1 protein (codons 1–66) with a CFP tag at the C terminus (M1-CFP). For visualization of the Golgi apparatus, we use codons 1–60 of human galactosyltransferase with a CFP tag at the C terminus (GalT-CFP). Fluorescent labeling of mitochondria can be achieved by expressing mCherry extended with the 33 C-terminal amino acids of Bcl-Xʟ or by the addition of 25 p*M* MitoTracker Red CMXRos (Molecular Probes) 10 min prior to imaging. One can readily perform triple color localization using MitoTracker Red CMXRos, as it can be resolved from both the YFP-tagged Ras and the CFP-tagged ER or Golgi marker. Cotransfection is as described earlier using SuperFect using a plasmid DNA ratio of 2:1 CFP-compartment marker:YFP-Ras.

Twenty-four hours post-transfection, cells containing the YFP-tagged protein with M1-CFP, GalT-CFP, and/or MitoTracker are imaged with a Zeiss 510 LSM using a metabolic chamber (e.g., Zeiss Incubator L-M) as described previously. Images are acquired before and at various times after addition of a PKC agonist. M1-CFP is absolutely restricted to the ER (Chiu *et al.*, 2002) and should appear as a reticular system of membranes contiguous with the nuclear envelope. In COS-1 cells, this reticular network often forms a more intense nexus in the paranuclear region also occupied by the Golgi apparatus and endosomal recycling compartment. GalT-CFP should appear as a paranuclear cluster of vesicles, tubules, and tubulovesicular structures. MitoTracker should appear as a tubulovesicular network that does not emanate from the nuclear membrane. The morphology of mitochondria often changes upon addition of a PKC agonist and these organelles can take on a more vesicular appearance as a consequence of fission. Colocalization of phosphorylated K-Ras to each compartment can be documented using confocal software to adjust the gains such that each fluorophore decorates organelles with relatively equal intensity. Under these conditions, a pseudocolor for colocalization (yellow in the case of red and green primary channels) indicates colocalization. However, one must exercise caution in not overinterpreting the yellow pseudocolor of the merge. For example, if one has a bright red mitochondrion that lies within a saturated area of green from any source, it will give a false-positive signal for colocalization on the basis of color. To avoid this, one should be careful to include morphology in the assessment of colocalization. That is, if phosphorylated YFP-Ras truly decorates mitochondria, one should see in the YFP channel a structure of identical size and shape to that made visible by MitoTracker Red CMXRos.

5. Targeting of PKC to Activated K-Ras with the Ras Binding Domain of Raf-1 (RBD)

Data indicate that translocation of K-Ras is regulated by a farnesyl-electrostatic switch that is in turn regulated by phosphorylation by PKC of serine 181. PKCs are activated by recruitment via their C1 domains to membranes rich in diacylglycerol (DAG). To confirm that PKC had this ability to phosphorylate K-Ras and affect its membrane association, we devised a method to target the catalytic domain of a PKC to activated Ras in a DAG-independent fashion.

The Ras binding domain of Raf-1 binds to GTP-bound Ras with 10^4 higher affinity than it binds GDP-bound Ras and has therefore been used as a reporter for Ras activation (Chiu *et al.*, 2002). We therefore sought to determine if the catalytic domain of a PKC (PKCθ) delivered to K-Ras via a fused RBD would have the ability to phosphorylate K-Ras and alter its subcellular localization.

5.1. Construction of pCGN-HA-RBD-PKCθcat

The construction of pCGN-HA-RBD-PKCθcat can be accomplished in three steps. Vectors are available commercially with robust multiple cloning sites that add a coding sequence of interest in-frame with an HA tag. We use pCGN for this purpose, which has only a single cloning site, *Bam*HI. To facilitate cloning into this site, we first create a fusion protein in pEGFP consisting of the RBD and the catalytic domain of PKCθ (PKCθcat). First, the cDNA for the RBD is PCR amplified with 5′-*Eco*RI and 3′-*Apa*I linkers designed into the primers. The amplification product is ligated in-frame into the pEGFP-C1 vector, which has been linearized by double digestion with *Eco*RI and *Apa*I and purified. Second, the cDNA for the catalytic domain of PKCθ (PKCθcat) is PCR amplified with 5′-*Apa*I and 3′-*Bam*HI linkers designed into the primers. The amplification product is then ligated in-frame into the vector pEGFP-C1-RBD, which has been linearized by double digestion with *Apa*I and *Bam*HI and purified. Third, the coding sequence for RBD-PCKθcat is PCR amplified from the pEGFP-C1-RBD-PKCθcat vector with 5′- and 3′-*Bam*HI linkers designed into the primers. Note also that the 3′ primer has a stop codon inserted before the *Bam*HI linker. This amplification product could then be ligated into the pCGN-HA, which has been linearized by digestion with *Bam*HI and purified, to provide the desired product.

PCR primers for amplification of RBD and PKCθcat to produce pCGN-HA-RBD-PKCθcat:

Forward *Eco*RI RBD: 5′-tcgattctgccttctaagacaagcaacactatcc-3′
Reverse *Apa*I RBD: 5′-cagggccccaggaaatctacttgaagttcttctcc-3′

Forward *Apa*I PKCθcat: 5′-aacagggcccggcacaagatgttgggaaaagga-3′
Reverse *Bam*HI PKCθcat: 5′-cggtggatcccgggagcaaatgagagtctccat-3′
Forward *Bam*HI RBD-PKCθcat: 5′-tcggatccccttctaagacaagcaacactatcc-3′
Reverse *Bam*HI RBD-PKCθcat-STOP: 5′-cggtggatcccgtcaggagcaaatga-gagtctccat-3′

5.2. Transfection and analysis of localization

Twenty-four hours before transfection, 2.0×10^5 MDCK cells are seeded in 35-mm MatTek culture dishes in 2 ml DMEM, supplemented with 10% FBS and antibiotics. The cells should be grown to 70% confluence on the day of transfection. Two micrograms of pCGN-HA-RBD-PKCθcat cDNA can be cotransfected with 2 μg of either pEGFP-K-Ras12V181S or pEGFP-K-Ras12V181A plasmid DNA into MDCK cells with high efficiency using the SuperFect reagent as described earlier. After a suitable period of time has passed to ensure expression of the constructs, MDCK cells can be imaged live using a Zeiss 510 LSM. In cells expressing this construct, GFP-K-Ras12V with a wild-type C terminus is partially internalized but GFP-K-Ras12V181A is not. This result confirms that S181 is a PKC site and that its phosphorylation is necessary and sufficient for PKC-induced translocation of K-Ras to internal membranes.

6. Single-Cell Fluorescent Analysis of Apoptosis: YFP-Caspase Sensor

We observed that activated K-Ras with a phosphomimetic substitution at residue 181, K-Ras12V181E, induced cell death that could be blocked by overexpression of Bcl-2, suggesting apoptosis. Accordingly, we sought an assay that could quantify apoptosis in cells expressing this K-Ras allele and variations thereof. Numerous assays have been developed to assess apoptosis such as annexin-V or TUNEL staining or poly (ADP-ribose) polymerase (PARP) cleavage. One disadvantage of these assays is that they measure apoptosis in a population of cells. To study apoptosis induced by expression of a transfected gene, such as K-Ras with a phosphomimetic substitution at position 181, we sought a fluorescence-based assay that would read out specifically in cells transfected with and expressing exogenous genes. A commercially available fluorescent probe for caspase-3 activation meets these criteria. Monitoring caspase-3 activation is a valuable tool for detecting apoptosis downstream of both extrinsic and intrinsic pathways of programmed cellular death.

The pCaspase3-Sensor vector (BD Clontech) encodes the enhanced yellow-green variant (EYFP) of GFP fused at the 3′ end to three copies of the nuclear localization signal (NLS) of the simian virus 40 large T-antigen. At the 5′ end, the gene contains a sequence encoding the nuclear export signal (NES) of the MAP kinase kinase (MAPKK) followed by a 36 nucleotide sequence encoding the region of PARP cleaved by caspase-3. NES is functionally dominant over the NLS such that the full-length fluorescent fusion protein distributes homogeneously to the cytosol in healthy cells. If caspase-3 is activated, it cleaves the PARP substrate, removing the NES and allowing the truncated EYFP-NLS fusion to enter the nucleus with high efficiency. Translocation of the fluorescent protein from the cytosol to the nucleus, which is detected easily by fluorescence microscopy, indicates caspase-3 activity at the single-cell level. One can cotransfect pCaspase3-Sensor along with the gene of interest in a 1:5 plasmid DNA ratio to make sure that fluorescent cells to be scored for apoptosis are cotransfected. This offers a great advantage over methods that report apoptosis over the entire population of cells, those that are transfected and those that are not.

6.1. Transfection and scoring of fluorescence

Twenty-four hours before transfection, 2.0×10^5 COS-1 cells are seeded in 35-mm glass bottom culture dishes in 2 ml DMEM, supplemented with 10% FBS and antibiotics. The cells should be grown to 70% confluence on the day of transfection. Although almost any cell type can be used in conjunction with the pCaspase3-Sensor to study the effects of a protein of interest on apoptosis, COS-1 cells were chosen for several reasons. First, COS-1 cells are transfected easily; they readily take up plasmid DNA transfected with common reagents, such as SuperFect or Lipofectamine, with typical transfection efficiencies surpassing 90%. Second, COS-1 cells generally express exogenous proteins expressed from plasmids incorporating an SV40 origin of replication to high levels, ensuring that both the experimental protein of interest and the YFP-caspase sensor are expressed to sufficient levels. Finally, and probably most critical for this assay, COS-1 cells spread well and therefore display a flat morphology that is favorable for scoring for nuclear translocation of the fluorescent probe.

The wild-type and mutated K-Ras alleles cloned into the mammalian expression vectors such as pCGN-HA could be transfected into COS-1 cells with high efficiency using the SuperFect reagent, as described earlier. Other proteins of interest in mammalian expression vectors can also be used to assess their effects on apoptosis. Two and a half micrograms of plasmid DNA for the K-Ras construct is cotransfected with 0.5 μg pCaspase3-Sensor DNA. This ratio of nonfluorescent K-Ras plasmid DNA to sensor DNA ensures that cells expressing EYFP are also highly likely to express the K-Ras

construct. A 1:1 ratio of plasmid DNA to sensor DNA can be used if the protein of interest is also fluorescently tagged with a protein that can be spectrally resolved from the YFP-tagged sensor (e.g., CFP or mCherry). In this format, only cells coexpressing both the fluorescent protein of interest and the pCaspase3-Sensor are scored for caspase-3 activation. Following transfection, cells are grown for 12 to 18 h at 37° in 5% CO_2 to allow expression of the constructs. Optimization of the expression period is necessary to visualize cells in the early stages of apoptosis where caspase-3 is activated, but the cells have not yet undergone drastic morphological changes.

Any epifluorescence microscope equipped with a YFP or FITC filter can be used to inspect the subcellular localization of pCaspase3-Sensor EYFP fluorescence. Although confocal microscopy works well, it is not necessary to obtain a clear result. We scan various quadrants of the MatTek plate and score cells visualized as having cytosolic, nuclear, or indeterminate fluorescence. It is important to score all cells as they are encountered to avoid bias. We typically collect data until we have scored 100 cells for which the fluorescent pattern is clearly either nuclear or cytosolic. For reasons that are unknown, nucleoli are fluorescent in both healthy and apoptotic cells and can appear quite prominent so it is important to avoid scoring cells as nuclear on the basis of nucleolar fluorescence. A cell should be scored as nuclear when the nucleoplasm (excluding nucleoli) is brighter than the cytosol. For each condition, an apoptotic index can be calculated as the percentage of fluorescent cells displaying nuclear fluorescence (Fig. 7.3).

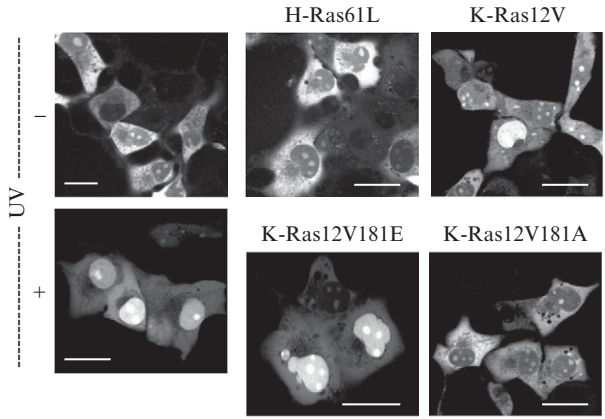

Figure 7.3 Single cell assay for apoptosis using a YFP caspase 3 sensor. COS-1 cells were transfected with pCaspase3-Sensor and imaged before and after exposure to UV radiation (left) or 12 h after cotransfection with the indicated K-Ras allele (right). Note that nucleoli are fluorescent in both healthy and apoptotic cells. Bars indicate 10 μm.

REFERENCES

Ballester, R., Furth, M. E., and Rosen, O. M. (1987). Phorbol ester- and protein kinase C-mediated phosphorylation of the cellular Kirsten ras gene product. *J. Biol. Chem.* **262,** 2688–2695.

Bivona, T. G., Quatela, S. E., Bodemann, B. O., Ahearn, I. O., Soskis, M. J., Mor, A., Miura, J., Wiener, H. H., Wright, L., Saba, S. G., Yim, D., Fein, A., *et al.* (2006). PKC regulates a farnesyl-electrostatic switch on K-Ras that promotes its association with Bcl-XL on mitochondria and induces apoptosis. *Mol. Cell.* **21,** 481–493.

Chiu, V. K., Bivona, T., Hach, A., Sajous, J. B., Silletti, J., Wiener, H., Johnson, R. L., Cox, A. D., and Philips, M. R. (2002). Ras signalling on the endoplasmic reticulum and the Golgi. *Nat. Cell Biol.* **4,** 343–350.

Choy, E., Chiu, V. K., Silletti, J., Feoktistov, M., Morimoto, T., Michaelson, D., Ivanov, I. E., and Philips, M. R. (1999). Endomembrane trafficking of ras: The CAAX motif targets proteins to the ER and Golgi. *Cell* **98,** 69–80.

Downward, J., Graves, J. D., Warne, P. H., Rayter, S., and Cantrell, D. A. (1990). Stimulation of p21ras upon T-cell activation. *Nature* **346,** 719–723.

Hancock, J. F., Paterson, H., and Marshall, C. J. (1990). A polybasic domain or palmitoylation is required in addition to the CAAX motif to loacalize $p21^{ras}$ to the plasma membrane. *Cell* **63,** 133–139.

Jackson, J. H., Li, J. W., Buss, J. E., Der, C. J., and Cochrane, C. G. (1994). Polylysine domain of K-ras 4B protein is crucial for malignant transformation. *Proc. Natl. Acad. Sci. USA* **91,** 12730–12734.

Leventis, R., and Silvius, J. R. (1998). Lipid-binding characteristics of the polybasic carboxy-terminal sequence of K-ras4B. *Biochemistry* **37,** 7640–7648.

McLaughlin, S., and Aderem, A. (1995). The myristoyl-electrostatic switch: A modulator of reversible protein-membrane interactions. *Trends Biochem. Sci.* **20,** 272–276.

Valge, V. E., Wong, J. G., Datlof, B. M., Sinskey, A. J., and Rao, A. (1988). Protein kinase C is required for responses to T cell receptor ligands but not to interleukin-2 in T cells. *Cell* **55,** 101–112.