Mutational and Biochemical Analysis of Plasma Membrane Targeting Mediated by the Farnesylated, Polybasic Carboxy Terminus of K-ras4B†

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Received March 6, 2000; Revised Manuscript Received May 5, 2000

ABSTRACT: Mutational analysis and in vitro assays of membrane association have been combined to investigate the mechanism of plasma membrane targeting mediated by the farnesylated, polybasic carboxy-terminal sequence of K-ras4B in mammalian cells. Fluorescence-microscopic localization of chimeric proteins linking the enhanced green fluorescent protein (EGFP) to the K-ras4B carboxy-terminal sequence, or to variant forms of this sequence, reveals that the normal structure of this targeting motif can be greatly altered without compromising plasma membrane-targeting activity so long as an overall strongly polybasic/amphiphilic character is retained. An EGFP/K-ras4B(171−188) chimeric protein was readily abstracted from isolated cell membranes by negatively charged lipid vesicles, and this abstraction was markedly enhanced by the anionic lipid-binding agent neomycin. Our results strongly favor a mechanism in which at the plasma membrane the carboxy-terminal sequence of K-ras4B associates not with a classical specific proteinaceous receptor but rather with nonspecific but highly anionic 'sites' formed at least in part by the membrane lipid bilayer. Our findings also suggest that the recently demonstrated prenylation-dependent trafficking of immature forms of K-ras4B through the endoplasmic reticulum [Choy et al. (1999) *Cell 98,* 69−80], while required for maturation of the protein, beyond this stage may not be essential to allow the ultimate delivery of the mature protein to the plasma membrane.

Low molecular weight G-proteins of the ras family play key roles in a variety of cellular regulatory processes and are found to be mutationally activated in a substantial proportion of human tumors (*1*−*4*). In mammalian cells, the mature forms of H- and N-ras, as well as the -4A and -4B isoforms of K-ras, are found predominantly associated with the plasma membrane, a localization that is important for both the normal and the oncogenic activities of these proteins (*4*−*7*). The plasma membrane localization of each of these ras proteins is determined by its farnesylated carboxy-terminal sequence (*5*−*7*), which can function as an autonomous plasma membrane-targeting signal when coupled to heterologous proteins (*8*−*11, 12*).

The plasma membrane-targeting signals of N-ras, H-ras, and K-ras4A comprise a carboxyl-terminal cysteine residue, which is farnesylated and carboxyl-methylated, preceded by a short amino acid sequence including one or more cysteine residues that serve as palmitoylation sites and which are required for correct subcellular targeting (*5, 6, 11*−*14*). By contrast, the plasma membrane-targeting signal of K-ras4B combines a farnesylated cysteine residue with a strongly polybasic sequence that lacks palmitoylation sites [-SKDGKK-KKKKSKTKC(farnesyl)-OCH_3]. Both the farnesylated cysteine residue and the adjacent polybasic sequence are required for normal plasma membrane localization of K-ras4B and to support the transforming activity of mutationally activated forms of the protein (*5, 7*).

To date, the mechanisms that establish and maintain the specific plasma membrane localization of K-ras4B remain largely undefined. Elucidation of these issues may be of broader interest in view of the occurrence of lipidated/polybasic motifs in other intracellular proteins (*15*−*17*) and of the potential that factors involved in K-ras4B targeting could offer novel loci for pharmacological intervention to inhibit the effects of oncogenically mutated forms of the protein. Partially processed forms of K-ras4B [terminating in either a -C(farnesyl)VIM or a -C(farnesyl)-OH structure] have been shown to associate with intracellular membranes (*11, 12, 18*) and with microtubules (*19*), in the former context at least in part to mediate the final steps in maturation of the protein's carboxy terminus. However, the mechanism by which the fully processed form of K-ras4B is subsequently recruited to the plasma membrane remains obscure. It is also unclear with what plasma membrane components the targeting sequence of K-ras4B interacts to maintain correct localization once the protein has reached this destination (*20*).

In the present study, we have combined an extensive mutational analysis with in vitro measurements of membrane association to investigate the mechanism of plasma membrane targeting mediated by the carboxy terminus of K-ras4B. To permit direct visualization of protein targeting mediated by the K-ras4B carboxy-terminal sequence in living cells, and to eliminate any possible contributions of other portions of the K-ras4B sequence to targeting, for these studies we have used chimeric proteins fusing the enhanced green fluorescent protein (EGFP)[1] to the carboxy-terminal sequence

† This work was supported by an operating grant from the Medical Research Council of Canada (Grant MT-7776) to J.R.S. and by a postdoctoral fellowship award to M.-O.R. from la Fondation pour la Recherche Médicale (Paris).

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10.1021/bi000512q CCC: $19.00
Published on Web 06/21/2000

of K-ras4B (amino acids 171−188), or to variant forms of this sequence.

MATERIALS AND METHODS

Preparation of Plasmids Encoding Chimeric Proteins. The coding sequences of all plasmid constructs described below were confirmed using the dideoxynucleotide method (Sequenase, Ver. 2.0, USB Corp.) and were inserted into the multiple cloning site of pcDNA3 (Invitrogen) for expression in mammalian cells. All expressed EGFP-containing chimeric proteins were observed to give single major bands upon analysis by SDS−PAGE and immunoblotting; all constructs incorporating a functional prenylation motif comigrated with the 'parent' EK chimera, indicating that they were prenylated (*13*).

Exon 4B of the K-*ras* gene, encoding the carboxy-terminal sequence of K-ras4B, was cloned by PCR from a human placental genomic library and subcloned into Bluescript II KS(+/−). This and plasmid pEGFP-C1 (Clontech) were used as templates for overlap-extension PCR to produce the sequence encoding the EK chimera (*21*). Mutations were introduced into the K-*ras*4B-derived portion of this sequence by oligonucleotide-directed mutagenesis using the Altered Sites system (Promega).

The EGFP sequence lacking the initiator methionine, ending in a TAA termination codon and flanked by *Bam*HI and *Xba*I sites, was obtained by PCR from pEGFP-C1 and inserted into the multiple cloning site of pcDNA3. A double-stranded oligonucleotide, containing the coding sequence 5′-ATGGGGAGTAGCAAGAGCAAAGATGGTAAAAA-GAAGAAAAAGAAGTCAAAGACAAAG-3′ flanked by 5′-*Kpn*I and 3′-*Bam*HI sites, was then inserted between these sites in the plasmid just described to generate the sequence encoding the SKE chimera. Sequences encoding the constructs AKE and AE were prepared by PCR mutagenesis using the SKE-encoding sequence as template.

Sequences encoding the Erap1a, $EAmph_8$, and EK_{AQ} constructs were generated by PCR from the EK-encoding sequence and inserted between the *Bam*HI and *Xba*I sites of pcDNA3. Mutant versions of the EK_{AQ} sequence, as well as the $EAmph_6$ sequence, were obtained by oligonucleotide-directed mutagenesis as described above.

Cell Transfection and Fluorescence Microscopy. Monolayers of CV-1 cells, grown to ca. 30% confluency on glass coverslips as described previously (*22*), were transfected by the calcium phosphate method (*23*). The monolayers were washed with fresh serum-containing medium 16 h after transfection and examined by microscopy after a further 48 h in culture. Live cell monolayers bathed in Hanks' buffered saline solution were visualized using an inverted epifluorescence microscope (EM35; Carl Zeiss, Inc., Thornwood, NJ) or a Zeiss 410 laser scanning confocal microscope. For each EGFP construct described, at least 100 chimera-expressing cells (from 3 independent transfections) were visually examined, and at least 30−50 were photographed. The micrographs shown in Figures 1−4 are representative of the balance of perinuclear, plasma membrane, and cytoplasmic/nuclear fluorescence in the majority (in all cases at least 90%) of the cells examined at the standard time of 3 days post-transfection. Cells examined at shorter times (36−48 h) after transfection with certain prenylated constructs showed a significantly greater fraction of diffuse intracellular (presumably cytoplasmic) fluorescence, the appearance of which was invariably accompanied by detection of an elevated fraction of the expressed protein in the soluble fraction upon fractionation and of multiple major bands upon electrophoresis/immunoblotting analysis, suggesting incomplete processing (*13*).

Cell Fractionation. CV-1 cell membranes were prepared as follows, performing all steps at 4 °C. Monolayers of cells transiently expressing the EK chimeric protein were washed twice with phosphate-buffered saline, incubated for 15 min in the same medium plus 2 mM EDTA, and removed from the substrate by scraping. After pelleting in the cold (1000*g*, 5 min), the cells harvested from 8 six-well or 100-mm culture dishes were resuspended in 1 mL of lysis buffer (20 mM sodium phosphate, 2 mM EDTA, pH 7.4, containing 10 μg/mL each aprotinin, leupeptin, and soybean trypsin inhibitor plus 2 mM phenylmethanesulfonyl fluoride), incubated for 20 min, and passed 30 times through a 27-gauge needle. The suspension was centrifuged (1000*g*, 10 min) to remove intact cells and nuclei, and $MgCl_2$ was added (as a concentrated stock) to a final concentration of 2 mM before centrifugation (250000*g*, 60 min). The resulting membrane pellet was resuspended in 2 mL of lysis buffer using a 27-gauge needle, $MgCl_2$ was again added to 2 mM, and the suspension was recentrifuged as above, and then resuspended in 800 μL of lysis buffer. The phospholipid content of membrane preparations used for incubation with liposomes (see below) was determined by lipid extraction (*24*) followed by perchloric acid digestion and assay of liberated phosphate (*25*). Membrane fractionation on Optiprep gradients and assay of protein and marker activities were carried out as described elsewhere (*26, 27*).

To determine the distributions of EGFP-containing chimeric proteins between the sedimentable and soluble fractions of CV-1 cells, the above fractionation was carried out on a one-eighth scale, and aliquots of the pellet and supernatant fractions were analyzed by immunoblotting as described below. Fractionation data are reported only for species showing virtually complete association with either the membrane or the cytoplasmic fraction. As discussed previously (*11*), because of the enormous dilution of cell components upon cell disruption, the cell-fractionation assay systematically underestimates the extent of in vivo membrane association for proteins that bind membranes with low to moderate affinity.

Membrane/Liposome Coincubations. All phospholipids used for liposome preparation were 1-palmitoyl-2-oleoyl-species purchased from Avanti Polar Lipids (Alabaster, AL). Lipids [including 0.025% rhodaminyl-phosphatidylethanolamine (rhodaminyl-PE) as a marker] were dried down under nitrogen from chloroform/methanol, and then further dried under high vacuum for 8−12 h. Liposomes were prepared by first bath-sonicating the dried lipids in distilled water to near-clarity, then adding small volumes of concentrated buffer (to final concentrations of 100 mM NaCl, 20 mM phosphate, 2 mM EDTA, 50 mM lipid, pH 7.4), and

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid, trisodium salt; (E)GFP, (enhanced) green fluorescent protein; PC (PG), 1-palmitoyl-2-oleoyl-phosphatidylcholine (-phosphatidylglycerol); rhodaminyl-PE, *N*-lissamine rhodaminesulfonylphosphatidylethanolamine.

sonicating for a further 5 min. The resultant lipid vesicle dispersion was centrifuged at 4 °C, 250000*g*, for 60 min to remove a minor fraction of sedimentable lipid.

Liposomes prepared as just described were incubated (for 1 h except where otherwise indicated) with 40 μL of CV-1 cell membrane suspension (containing ca. 30 nmol phospholipid) in a total volume of 135 μL adjusted to a final composition of 100 mM NaCl, 1 mM 'free' $MgCl_2$ (excess over EDTA), 20 mM phosphate, pH 7.4. Sucrose was then added (as a 50% w/v solution) to a final level of 5% (w/v), and the sample was centrifuged for 1 h at 170000*g* (22 °C). Aliquots of the supernatant and the resuspended pellet were analyzed on 10% SDS−PAGE gels, transferred to nitrocellulose, and immunoblotted using rabbit polyclonal anti-GFP antibody (Molecular Probes, Eugene, OR) or mouse monoclonal anti(Na,K-ATPase α-subunit) antibody (H6, a generous gift of Dr. Michael Caplan, Yale University) followed by the appropriate peroxidase-conjugated secondary antibody (goat anti-rabbit or anti-mouse, BioRad Laboratories, Mississauga, Ontario). Immunoblots were visualized by enhanced chemiluminescence (Renaissance reagent, NEN) and analyzed quantitatively by scanning densitometry (BioImage system, Millipore). Aliquots of the pellet and supernatant fractions were also analyzed for the distribution of lipid (monitored via rhodaminyl-PE fluorescence) using a Perkin-Elmer LS-5 spectrofluorometer (λ_{ex} = 525 nm, λ_{em} = 596 nm). To avoid artifacts due to possible contamination of the supernatant fraction by resuspended membrane material, in the above experiments the upper and lower halves of the supernatant were recovered and analyzed separately for content of the EK chimera and of lipid vesicles. Estimates reported for the proportion of the EK chimera in the supernatant fraction were calculated as follows:

$$\text{(proportion of EK in supernatant)} = [f(\text{EK})_{\text{upper sup.}}]/[f(\text{Lipid})_{\text{upper sup.}}]$$

where $f(\text{EK})_{\text{upper sup.}}$ and $f(\text{Lipid})_{\text{upper sup.}}$ represent the measured proportions of total chimera and of total liposomal lipid, respectively, in the upper supernatant fraction. Parallel estimates, calculated by simply summing the proportions of total chimeric protein in the two supernatant fractions, in most cases agreed closely with estimates determined as just described.

RESULTS

Mutational Analysis of the K-ras4B Plasma Membrane-Targeting Sequence. CV-1 cells were transiently transfected to express chimeras linking EGFP to the wild-type and variant forms of the K-ras4B carboxy-terminal sequence (residues 171−188). The sequences and designations of the chimeras examined are indicated in Table 1. The distributions of these chimeric proteins were examined in living cells by fluorescence microscopy and, for some species, by subcellular fractionation as well.

As shown in Figure 1A, an EGFP/K-ras(171−188) chimera (hereafter designated EK) shows a high degree of plasma membrane targeting in CV-1 cells, similar to results obtained with other chimeric proteins bearing this targeting sequence (*8*−*10*, *28*). Upon fractionation of the cells into soluble and sedimentable fractions, the chimeric protein was recovered almost completely in the latter fraction (Table 2). The specific localization of this construct to the plasma membrane was confirmed by confocal microscopy, as shown in Figure 3A. By contrast, cells expressing unmodified EGFP show a diffuse fluorescence in both the cytoplasm and the nucleus (Figure 1B), and the protein was found entirely in the soluble fraction upon cell fractionation (Table 2). A similar cytoplasmic/nuclear localization results when the farnesylated cysteine residue in the EK chimera is mutated to serine (Figure 1F and Table 2), in agreement with previous findings for K-ras4B itself and a (protein A)/K-ras4B(171−188) chimera (*5*, *6*).

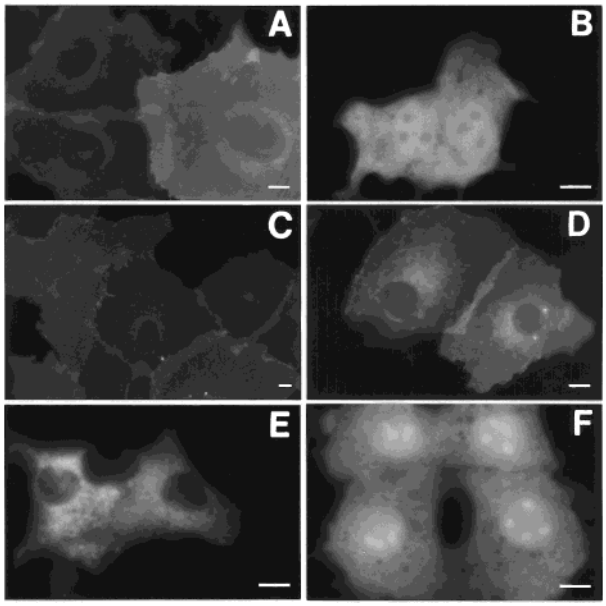

FIGURE 1: Both the polybasic sequence and the farnesyl residue of the K-ras4B carboxy-terminal sequence critically influence the plasma membrane targeting of EGFP/(K-ras4B carboxy-terminus) chimeras. Fluorescence micrographs obtained for live CV-1 cells transiently expressing the chimeric proteins (A) EK (wild-type K-ras4B targeting sequence), (C) Δ2K, (D) K3Q, (E) K6Q, or (F) EK(SVIM). Panel B, cells expressing unmodified EGFP. Conditions used for cell transfection and fluorescence microscopy are described under Materials and Methods. Space bar = 10 μm.

Table 1: Sequences of Targeted EGFP Constructs

Chimera	Targeting Sequence
EK	- S K D G K K K K K K S K T K C V I M
EK(CAIL)	- S K D G K K K K K K S K T K C A I L
EK(SVIM)	- S K D G K K K K K K S K T K S V I M
EK(K3Q)	- S K D G K Q K Q K Q S K T K C V I M
EK(K6Q)	- S K D G Q Q Q Q Q Q S K T K C V I M
EK(Δ2K)	- S K D G K K - - K K S K T K C V I M
EK_{AQ}	- A A A G K K K K K K A Q A Q C V I M
EK_{AQ}(K2Q)	- A A A G K K Q Q K K A Q A Q C V I M
EK_{AQ}(2QIns)	- A A A G K K K K Q Q K K A Q A Q C V I M
EK_{AQ}(K3Q)	- A A A G K Q Q Q K K A Q A Q C V I M
EK_{AQ}(3QIns)	- A A A G K K K Q Q Q K K K A Q A Q C V I M
$EAmph_8$	- A Q A G K K F W K R L R K F L R K L K S
$EAmph_6$	- A Q A G K K F W K R L R K F L R K A D S
AKE	m G L T V S A S K D G K K K K K K S K T K G -
AE	m G L T V S A -
SKE	m G S S K S K D G K K K K K K S K T K G -
Erap1a	- N A V K K K P K K K S C L L L

Table 2: Membrane Association of EGFP Chimeras[a]

construct	% in P100 fraction
EK	92 ± 2
EGFP	0
EK(SVIM)	5 ± 4
EK(GerGer)	98 ± 1
AKE	94 ± 2
SKE	97 ± 2
$Eamph_8$	98 ± 1

[a] CV-1 cells transiently expressing the indicated EGFP constructs were fractionated, and aliquots of the soluble and sedimentable fractions were analyzed by SDS−PAGE and immunoblotting using anti-GFP primary antibody, as described under Materials and Methods. Values shown (±SD) for all constructs were determined by duplicate measurements in each of at least two independent experiments.

Previous studies have found that the hexalysine sequence near the carboxy terminus of K-ras4B is important for plasma membrane targeting; while up to three of the six contiguous lysine residues, in various combinations, can be mutated to glutamine without detectable effects on plasma membrane localization, mutation of additional lysine residues leads to a gradual redistribution of K-ras4B to other intracellular loci (*5*, *7*, *11*). Similar results are observed for EGFP/K-ras4B-(171−188) chimeras. A chimera in which two of the six contiguous lysine residues are deleted appears to be as well-targeted to the plasma membrane as is the 'wild-type' EK chimera (Figure 1C). However, mutation of three of the contiguous lysine residues to glutamine leads to a slight but detectable redistribution of EGFP fluorescence to intracellular (notably perinuclear) membranes (Figure 1D). Mutation of all six lysine residues leads to a massive redistribution of fluorescence to intracellular membranes (Figure 1E), as Choy et al. (*11*) have also reported.

To examine the importance of amino acid residues surrounding the hexalysine motif to the targeting function of the K-ras4B(171−188) sequence, we mutated all of these residues except the flanking glycine and the farnesylation motif to produce the chimera EK_{AQ}. As shown in Figure 2A, this chimeric construct is targeted to the plasma membrane as specifically as is the chimera containing the wild-type K-ras4B targeting sequence (compare Figure 1A), indicating that the sequences flanking the hexalysine motif are not critical for plasma membrane targeting. However, when two of the six contiguous lysine residues within the EK_{AQ} targeting sequence are additionally mutated to neutral glutamine residues [construct EK_{AQ}(K2Q)], the specificity of plasma membrane localization is significantly reduced (Figure 2B), while, as noted above, deletion of two of the contiguous lysine residues within the wild-type K-ras4B targeting sequence does not discernibly alter the specificity of plasma membrane targeting (Figure 1C). Similarly, while as noted above substitution of three lysine residues by glutamine within the wild-type targeting sequence only modestly affects the specificity of plasma membrane targeting (Figure 1D), a similar substitution within the EK_{AQ} targeting sequence profoundly reduces the latter's plasma membrane-targeting activity (Figure 2C,D). These observations indicate that the regions flanking the hexalysine sequence also make a modest contribution to the plasma membrane-targeting activity of the K-ras4B carboxy-terminal region, which, however, is redundant if the hexalysine 'core' is preserved intact. This contribution may reflect the fact that the flanking sequences contribute an additional net charge of +2 to the targeting sequence.

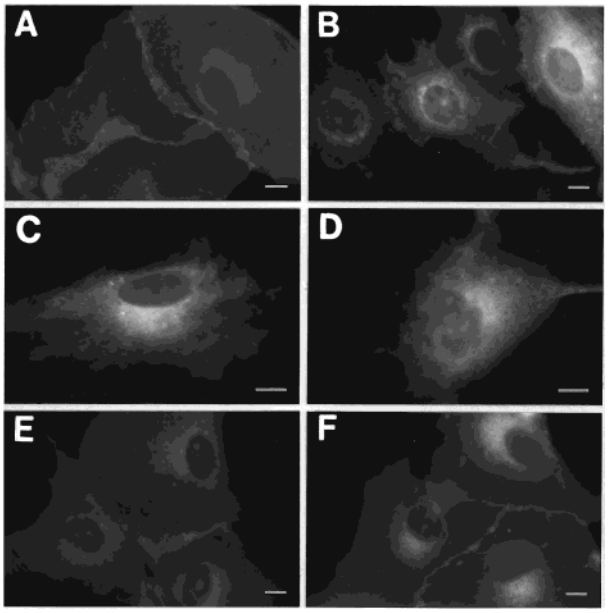

FIGURE 2: Net charge and not the spatial distribution of charges within the K-ras4B targeting sequence is crucial for plasma membrane targeting. Fluorescence micrographs shown were obtained for live CV-1 cells transiently expressing the chimeras (A) EK_{AQ}, (B) EK_{AQ}(K2Q), (C, D) EK_{AQ}(K3Q), or (E, F) EK_{AQ}(3QIns). Space bar = 10 μm. Conditions used for cell transfection and fluorescence microscopy were as described under Materials and Methods.

The hexalysine motif of K-ras4B could in principle promote specific plasma targeting either by virtue of its exact sequence, presenting a particular spatial arrangement of positive charges that is required for interaction with the targeting locus, or, in a less specific manner, simply by contributing to the high net positive charge of the targeting sequence. To distinguish between these two possibilities, we examined the plasma membrane targeting of EGFP-containing chimeras incorporating distinct but related variations of the K-ras4B hexalysine sequence. In these chimeras, the amino acid residues flanking the hexalysine core were mutated as in the above EK_{AQ} construct to increase the sensitivity of targeting to even small perturbations of the hexalysine sequence. As noted above, the chimera EK_{AQ}-(K3Q), in which three residues of the core hexalysine sequence are mutated to glutamine residues, shows very little specific association with the plasma membrane (Figure 2C,D). By contrast, the construct EK_{AQ}(3QIns), in which three glutamine residues are inserted into the hexalysine motif while retaining the full (+6) net charge of this sequence, is localized to the plasma membrane almost as specifically as is the 'parent' EK_{AQ} construct (Figure 2E,F). Similarly, a construct inserting two glutamine residues within the hexalysine segment of the EK_{AQ} targeting sequence [EK_{AQ}-(2QIns), not shown] was localized to the plasma membrane almost as specifically as the parent EK_{AQ} construct and with markedly greater specificity than that observed for the EK_{AQ}-(K2Q) construct (Figure 2B). This pattern of results appears inconsistent with a model postulating specific recognition of the hexalysine sequence by a proteinaceous targeting receptor but resembles that observed previously for binding of polybasic sequences to negatively charged lipid bilayers,

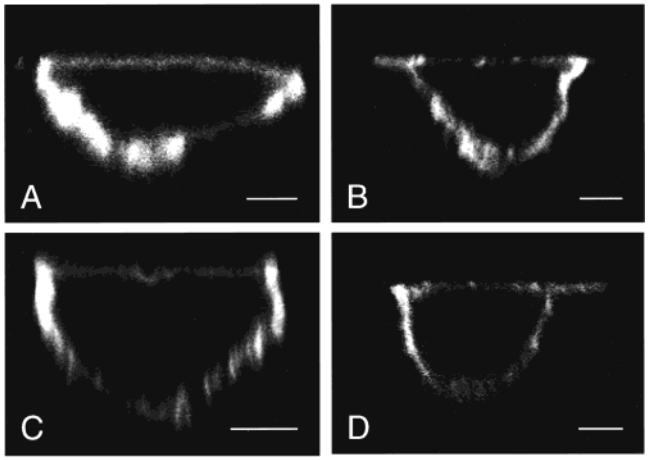

FIGURE 3: Confocal scanning fluorescence micrographs (*xz*-sections) obtained for live CV-1 cells transiently expressing the chimeric proteins (A) EK, (B) EK(GerGer), (C) AKE, or (D) Eamphi$_8$. Space bar = 10 μm. Details of cell transfection and fluorescence microscopy were as described under Materials and Methods.

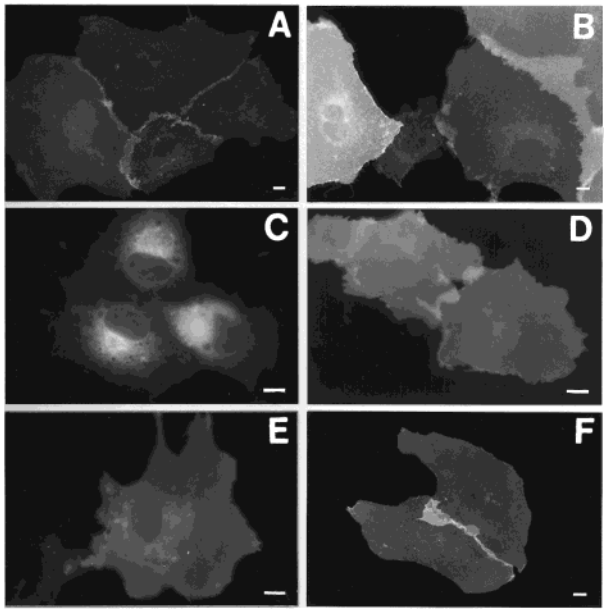

FIGURE 4: Various combinations of basic and hydrophobic residues can support the plasma membrane-localizing function of the K-ras4B targeting sequence. Micrographs were obtained for live CV-1 cells expressing the chimeras (A) EK(CAIL), (B) AKE, (C) AE, (D) SKE, (E) Erap1a, and (F) Eamphi$_8$. Space bar = 10 μm. Conditions for cell transfection and fluorescence microscopy were as described under Materials and Methods.

where binding affinity is only modestly weakened by the insertion of uncharged polar residues within the polybasic sequence (*29*) but is strongly attenuated by the loss of three positive charges (*30, 31*).

To determine whether the farnesyl group of K-ras4B contributes to plasma membrane targeting by virtue of its specific structure or simply its hydrophobic nature, we examined the localization of EGFP chimeras combining the K-ras4B polybasic sequence with alternative lipidic residues. Replacement of the farnesylation motif in the K-ras4B targeting sequence (-CVIM) with a geranylgeranylation motif derived from a brain Gγ subunit (-CAIL) causes no discernible change in the specificity of plasma membrane targeting of the protein, as assessed by either conventional (Figure 4A) or confocal fluorescence microscopy (Figure 3B). Similar results have been reported previously for K-ras4B (*6*). In a more dramatic modification, the polybasic region of the K-ras4B targeting sequence was linked to the myristoylation sequence of Arf5 and grafted to the N-terminus of EGFP (chimera AKE). Both conventional and confocal fluorescence microscopy (Figures 4B and 3C, respectively) demonstrated that the myristoylated construct was targeted to the plasma membrane with a high degree of specificity. By contrast, a construct (AE) in which EGFP was fused to the myristoylation sequence of Arf5 alone was largely associated with intracellular membranes (Figure 4C), consistent with the reported absence of any plasma membrane-targeting signal in Arf5 (*32*). Highly specific plasma membrane targeting was also observed for the chimeric construct SKE, in which the polybasic sequence of K-ras4B was combined with the myristoylation signal of v-src (Figure 4D). Both the AKE and SKE constructs, as well as the geranylgeranylated construct discussed above, were recovered almost entirely in the sedimentable fraction after cell fractionation and immunoblotting (Table 2). The high specificity of plasma membrane localization observed for the AKE and SKE chimeras is comparable to that observed for the 'wild-type' EK chimera [and significantly greater than that observed previously (*33*) for a farnesylation-deficient mutant of K-ras4B to which an N-terminal myristoylation signal was fused], indicating that neither the specific structure of the prenyl residue nor the C-terminal position of the K-ras4B polybasic/amphiphilic sequence is essential for the efficient plasma membrane-targeting function of this sequence.

The above results suggest that the plasma membrane incorporates sites with a promiscuous affinity for binding diverse amphiphilic/polybasic sequences. To explore this hypothesis further, we examined the plasma membrane targeting of two additional EGFP chimeras. The first (Erap1a), linking EGFP to the polybasic/geranylgeranylated carboxy-terminal sequence of rap1a, was preferentially targeted to the plasma membrane (Figure 4E), with a degree of specificity comparable to that observed for the EK$_{AQ}$ construct (Figure 2A), whose polybasic sequence carries the same net positive charge (+6). This finding is particularly striking given that rap1a itself has been reported to be localized to the Golgi apparatus (*17*), suggesting that the plasma membrane-targeting information specified by the protein's carboxy terminus may normally be overriden by signals encoded elsewhere within the rap1a sequence.

A final chimeric construct examined (EAmph$_8$) linked the EGFP sequence via its carboxy terminus to an entirely artificial sequence designed to form an amphiphilic α-helix with the same net charge as the K-ras4B targeting sequence (+8). This unlipidated chimeric species was observed to be targeted with high selectivity to the plasma membrane by both conventional (Figure 4F) and confocal fluorescence microscopy (Figure 3D). Cell fractionation and immunoblotting confirmed that the expressed chimeric protein was essentially completely associated with the cellular membrane fraction (Table 2). A related construct (EAmph$_6$) bearing an amphiphilic helix of net charge +6 was also targeted preferentially to the plasma membrane (not shown).

Abstraction of the EGFP/K-ras(171−188) Chimera from Isolated Cellular Membranes. To investigate further the

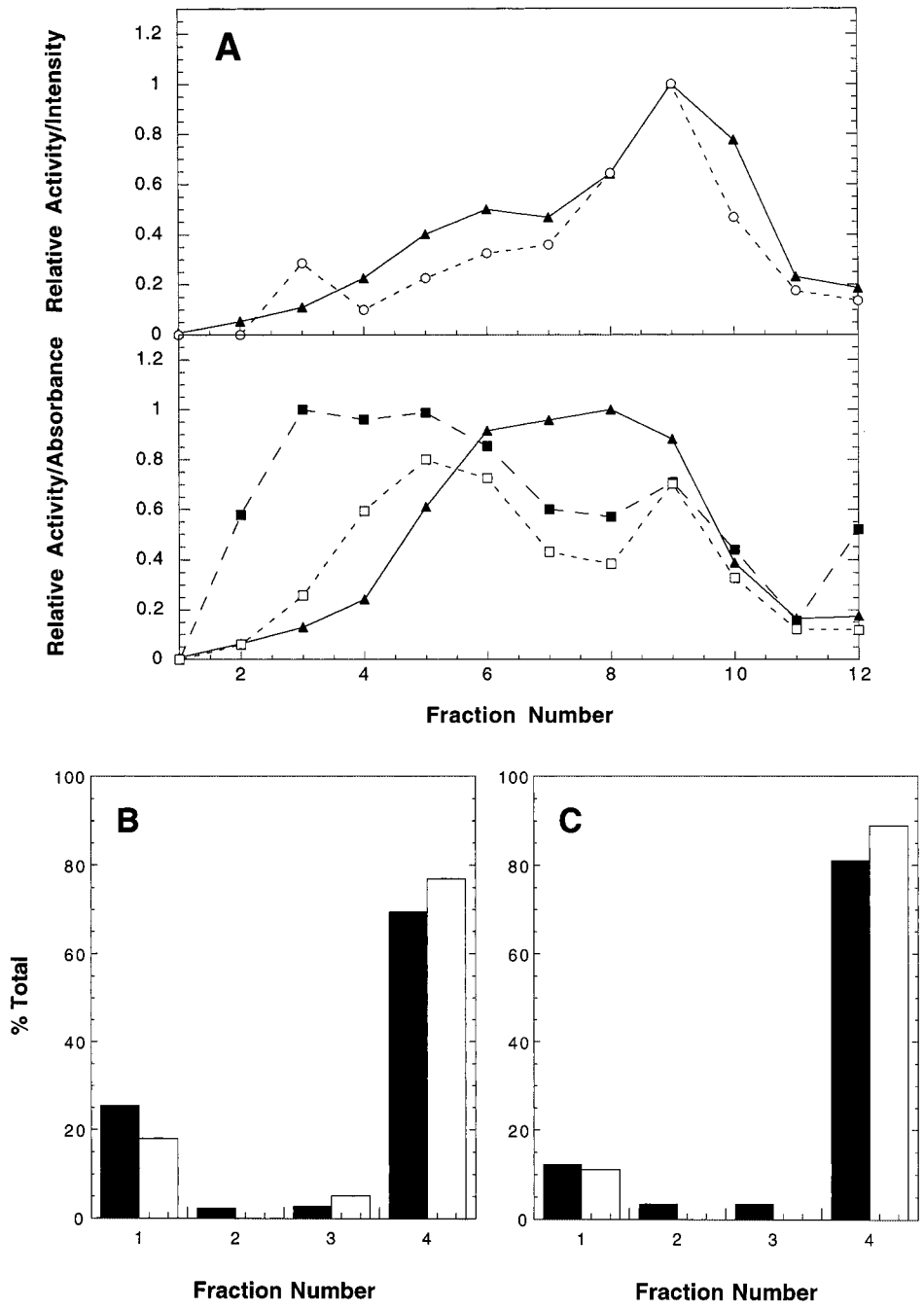

FIGURE 5: Panel A: Density-gradient fractionation of the high-speed total membrane pellet obtained from CV-1 cells and used for in vitro studies of the release of the EGFP/K-ras(171−188) chimera (EK) from cell membranes as described in the text. Upper panel: distributions of (○) the EK chimera and (▲) alkaline phosphatase activity; lower panel: distributions of (■) total protein, (▲) α-mannosidase II activity (Golgi marker), and (□) α-glucosidase activity (endoplasmic reticulum marker). Protein and marker enzyme activities were determined by spectrophotometric and fluorometric assays, respectively, and levels of the EK chimera was quantitated by SDS−PAGE and immunoblotting, all as described under Materials and Methods. Panels B and C: The EGFP/K-ras(171−188) chimera released from isolated CV-1 cell membranes in the presence of lipid vesicles becomes vesicle-bound. After incubation of isolated membranes with phosphatidylcholine/phosphatidylglycerol vesicles labeled with rhodaminyl-PE, the mixture was centrifuged and the supernatant recovered, all as described under Materials and Methods. A portion of the supernatant was then mixed with sucrose to a final concentration of 25% (final volume 50 μL), overlaid with 140 μL of 20% buffered sucrose and 40 μL of buffer, and centrifuged for 1 h at 170000*g*. The gradient was then recovered in four fractions (top = fraction 4) and assayed for rhodaminyl-PE fluorescence (liposomal marker) and chimera immunoblotting intensity as described under Materials and Methods. In panel B, membranes were incubated with 10 mM 75:25 (molar proportions) phosphatidylcholine/phosphatidylglycerol vesicles, and in panel C, membranes were incubated with 10 mM 85:15 (molar proportions) phosphatidylcholine/phosphatidylglycerol vesicles in the presence of 2 mM neomycin.

nature of the interaction between the K-ras4B carboxy-terminal sequence and the plasma membrane, we examined the effects of various treatments in vitro on the association of the EGFP/K-ras4B(171−188) chimera (EK) with isolated cell membranes. These experiments were carried out using a high-speed pellet fraction prepared from EK-expressing CV-1 cells as described under Materials and Methods. As shown in Figure 5A, when this fraction was centrifuged on a 4−30% Optiprep gradient the distribution of the EK chimera was similar to that of the plasma membrane marker

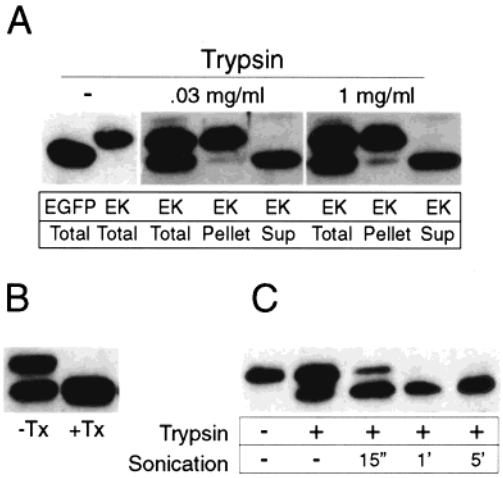

FIGURE 6: Trypsin digestion of the EK chimeric protein in isolated CV-1 cell membranes. (A) Membranes isolated from CV-1 cells expressing the EK chimera were incubated for 1 h at 37 °C in the presence of the indicated concentrations of trypsin. Aliquots of the total digest, and of the pellet and supernatant obtained upon subsequent centrifugation at 170000*g*, were analyzed by SDS−PAGE followed by immunoblotting with anti-GFP antibody as described under Materials and Methods. The sample in the leftmost lane was obtained by detergent solubilization of cells expressing unmodified EGFP. The immunoblots for trypsin-treated samples are imaged using a longer exposure to visualize minor components. (B) Isolated membrane samples from EK-expressing cells were treated with trypsin (1 mg/mL) in the presence or absence of 0.02% Triton X-100, and the digested samples were analyzed as described above. (C) Membrane samples from EK-expressing cells were incubated with or without trypsin (1 mg/mL), with or without prior bath-sonication for the indicated times, and then analyzed as described above.

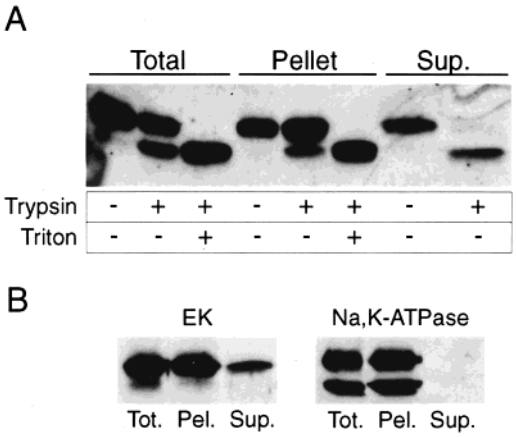

FIGURE 7: Anionic lipid-containing vesicles can abstract the EK chimeric protein from isolated CV-1 cell membranes. (A) The sedimentable fraction isolated from CV-1 cells expressing the EK chimera was incubated with 75:25 PC/PG vesicles (10 mM) for 1 h at 37 °C, and half of the incubation mixture was then centrifuged (170000*g*, 1 h). Aliquots of the unfractionated incubation mixture and of the subsequently isolated pellet and supernatant fractions were incubated with and without trypsin (1 mg/mL) and Triton X-100 (0.02% w/v), as indicated, and then analyzed by SDS−PAGE and immunoblotting with anti-GFP antibody. (B) The sedimentable fraction isolated from EK-expressing CV-1 cells was incubated with 75:25 PC/PG vesicles (10 mM), and then centrifuged as in (A). Replicate aliquots of the original incubation mixture and the subsequently isolated pellet and supernatant fractions were analyzed by SDS−PAGE followed by immunoblotting with either anti-GFP or anti-(Na,K-ATPase) α-subunit antibody, as indicated. Immunoblots are shown using relatively long exposures to permit visualization of even minor bands. The lower (and, as seen in shorter exposures, more intense) band in the anti-(Na,K-ATPase) immunoblots migrated at the normal position of the α-subunit (ca. 98 kDa). The slower-migrating band is an alternate form of the α-subunit (possibly an $\alpha\beta$ dimer) obtained under some conditions of sample preparation for SDS−PAGE (*51*). Other experimental details were as described under Materials and Methods.

alkaline phosphatase and markedly different from that of total protein or of markers for the endoplasmic reticulum (α-glucosidase) or Golgi (α-mannosidase II), indicating that the specificity of plasma membrane association of the protein was maintained in the isolated membranes.[2]

For the interpretation of the experiments described below, it was important to distinguish chimeric protein molecules exposed to the incubation medium from molecules sequestered within resealed membrane vesicles. This was readily accomplished by limited trypsin digestion. As illustrated in Figure 6A, treatment of the isolated membrane fraction with trypsin (1 h, 37 °C) converted a fraction of the EK molecules to a faster-migrating form. The reduction in size of the digested protein to that of unmodified EGFP [from an apparent molecular mass of 30.0 ± 0.1 kDa to 26.3 ± 0.3 kDa (mean ± SD of three experiments)] and the resistance of expressed EGFP itself to trypsin cleavage under the same conditions (not shown) suggest that the cleavage event entails the removal of most or all of the lysine-rich K-ras4B-derived targeting sequence from the EK chimera. In agreement with this finding, upon centrifugation following trypsin treatment, >95% of the cleaved form of the EK chimera was found in the supernatant while all of the uncleaved form remained in the membrane pellet (Figure 6A). The extent of cleavage of the EK chimera was not changed by varying the trypsin concentration 30-fold but increased to 100% when samples were mixed with as little as 0.02% Triton X-100 (giving a roughly equimolar ratio of detergent to lipid, a subsolubilizing level) or briefly bath-sonicated during incubation with trypsin (Figure 6B,C). These findings suggest that the trypsin-resistant fraction of the EK chimera represents protein molecules sequestered within sealed membrane vesicles, which become accessible to trypsin only when the membrane barrier is disrupted. In different membrane preparations, the proportion of the EK chimera that was trypsin-accessible varied from roughly 30% to almost 100%, presumably reflecting differences in the extent and/or the topology of resealing of the plasma membrane fraction.

When membranes isolated from cells expressing the EK chimera were incubated with negatively charged lipid vesicles for 1 h at 37 °C, then mixed with sucrose to 5% (w/v), and recentrifuged to pellet the membrane but not the vesicle fraction, a portion of the chimeric protein was found to remain in the supernatant (Figure 7A). Varying the period of membrane/vesicle incubation from 20 min to 10 h had no significant effect on the amount of chimeric protein abstracted by the lipid vesicles (not shown). When the vesicle-containing supernatant obtained in this manner was mixed with sucrose to a final concentration of 25%, layered under buffered 20% and 0% sucrose solutions, and recentrifuged at 170000*g* for 1 h, the chimeric protein codistributed with the lipid vesicles (Figure 5B). Conversely, when

[2] The small peak of chimera observed in higher-density fractions was consistently less than 10% of total chimera in replicate fractionations and may represent incompletely processed chimera bound to internal membranes (*11*, *12*), microtubules (*19*), or other non-plasma membrane structures.

membranes and lipid vesicles were coincubated as above but then centrifuged in the complete absence of sucrose, the upper portion of the supernatant was almost completely depleted of both vesicles and the released EK chimera (not shown). These findings together indicate that the chimera molecules abstracted from the membrane became associated with the lipid vesicles.

As illustrated in Figure 7A, the pool of EK chimera that remained in the supernatant after incubation with phosphatidylcholine/phosphatidylglycerol (PC/PG) vesicles was completely accessible to trypsin, while the pellet was considerably enriched in the trypsin-resistant fraction. These findings suggest that the trypsin-accessible but not the trypsin-resistant pool of the EK chimera can be transferred from isolated cell membranes to negatively charged lipid vesicles. Immunoblotting with antibody directed against the (Na,K)-ATPase α-subunit (Figure 7B) showed that this integral plasma membrane protein was found entirely in the pellet fraction after incubation of isolated membranes with lipid vesicles, confirming that the EK chimera was transferred to vesicles in molecular form and not as plasma membrane fragments.

In Figure 8A is plotted the extent of translocation of the EK chimera to the nonsedimentable fraction (expressed as a percentage of the trypsin-accessible pool) after incubation with varying concentrations of either 75:25 or 100:0 (molar proportions) PC/PG vesicles. As indicated (filled symbols), 75:25 PC/PG vesicles readily abstracted the chimeric protein from the membranes; the maximal amount of the EK chimera that could be abstracted approached 100% of the trypsin-accessible fraction. In contrast, vesicles composed purely of PC (open symbols) showed very little ability to abstract the chimeric protein from isolated membranes even at 20 mM lipid. These results agree with previous findings that the binding affinity of the K-ras4B targeting sequence for lipid vesicles increases greatly as the level of anionic lipid in the vesicles increases (*34*, *35*). PC/phosphatidylserine (75:25) vesicles abstracted the chimeric protein from the membranes as efficiently as did 75:25 PC/PG vesicles (not shown), consistent with our previous finding that vesicles with similar surface charge densities but distinct lipid compositions bind the targeting sequence of K-ras4B with similar affinities (*35*). Vesicles containing >25 mol % anionic lipid also readily abstracted the trypsin-accessible pool of the EK chimera from isolated cell membranes (not shown) but showed a greater tendency to adhere to and/or to mix lipids with cellular membranes and were thus not routinely used. In no case did the proportion of EK chimera extracted by vesicles exceed the proportion that was trypsin-accessible.

In a final series of experiments, we examined the effects of neomycin, a cationic trisaccharide derivative that binds at low millimolar concentrations to anionic lipids and with particular affinity to phosphoinositides (*36*), on the release of the EK chimera from cell membranes in the presence of liposomes. For these experiments, we used lipid vesicles containing low proportions of PG in PC, which in the absence of neomycin abstracted the EK chimera from cell membranes much less efficiently than did vesicles containing 25 mol % PG. As illustrated in Figure 8B, the addition of 2 mM neomycin caused a marked (roughly 2-fold) increase in the extent of release of the chimeric protein from isolated cell membranes in the presence of 95:5 PC/PG vesicles (10 mM); 0.5 mM neomycin also detectably enhanced the release of

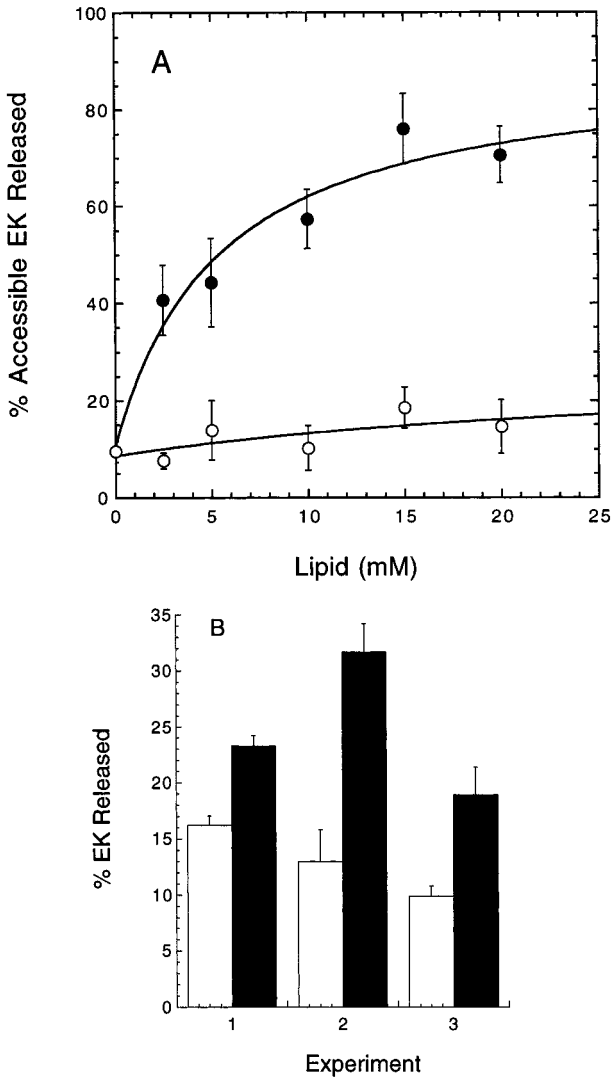

FIGURE 8: (A) Release of the EK chimera from isolated CV-1 cell membranes in the presence of phosphatidylcholine/phosphatidylglycerol (75:25) vesicles (filled symbols) or phosphatidylcholine vesicles (open symbols). Cell membranes isolated from EK-expressing cells were incubated with lipid vesicles at the indicated concentrations for 1 h at 37 °C, and the incubation mixtures were centrifuged (170000*g*, 1 h) to pellet the membrane but not the liposomal fraction. The contents of the EK chimera in each fraction were determined by SDS−PAGE and immunoblotting as described under Materials and Methods. Data shown represent the mean (±SEM) of duplicate determinations in four independent experiments. Fitting the data obtained using 75:25 phosphatidylcholine/phosphatidylglycerol vesicles to a rectangular-hyperbolic equation (solid curve) indicated that half-maximal abstraction occurs at a vesicle lipid concentration of 5.5 ± 0.8 mM and that a maximum of 92 ± 11% of the trypin-accessible fraction of EK molecules can be abstracted from the membranes. (B) Membranes isolated from cells expressing the EK chimera were incubated with 95:5 (molar proportions) PC/PG vesicles in the presence (solid bars) or absence (unshaded bars) of 2 mM neomycin, and then centrifuged, and the proportion of the chimeric protein released to the supernatant was determined as in (A). Data shown are the results of duplicate determinations in each of the three independent experiments presented.

the chimera to lipid vesicles (not shown). Neomycin also enhanced by a similar factor the extent of abstraction of the EK chimera from membranes in the presence of vesicles containing 15 mol % PG (not shown). The EK chimera released in the presence of neomycin codistributed with the lipid vesicles upon flotation of the supernatant fraction

through sucrose (Figure 5C), indicating that the chimera released under these conditions was again lipid vesicle-bound.

DISCUSSION

Our findings using EGFP chimeras agree with previous reports that the plasma membrane-targeting function of the K-ras4B carboxy-terminal region depends absolutely on both its amphiphilic and its strongly polybasic character (*5*−*7*). The present results suggest, however, that the plasma membrane 'target locus' for the K-ras4B carboxy-terminal sequence constitutes an exceedingly nonspecific binding site, exhibiting only the most generic requirements for an amphiphilic and highly polybasic protein sequence. Significantly, the structural selectivity of this binding locus, as revealed by our mutagenesis results, appears to be markedly different from that of a recently identified plasma membrane receptor which binds ras and other prenylated proteins in a strictly prenylation-dependent manner (*20*). While K-ras4B may of course also interact with this or other more 'classical' (i.e., specific) receptors at the plasma membrane, our results suggest that these latter interactions are not absolutely essential to support the plasma membrane-targeting function of the K-ras4B carboxy terminus.

Our present findings strongly suggest that the plasma membrane binding site for the K-ras4B targeting sequence is at least partly composed of membrane lipids. We base this conclusion on two main results. The first, as already noted, is our finding that strongly basic/amphiphilic motifs presenting radically different spatial arrangements of hydrophobic and basic residues can mediate equally efficient and specific plasma membrane targeting. These findings are difficult to reconcile with a model postulating a classical, well-defined protein binding site for the K-ras4B targeting sequence, but they are fully consistent with the known abilities of diverse polybasic/amphiphilic motifs to associate with negatively charged lipid bilayers. Second, we observe that release of the EGFP/K-ras4B(171−188) chimera from isolated CV-1 cell membranes to lipid vesicles is enhanced by neomycin, which binds to negatively charged lipids and with particular affinity to phosphoinositides (*36*), suggesting that the polybasic K-ras4B targeting sequence and neomycin are competing for association with such lipids at the plasma membrane.

Taking account of the above considerations, we can propose two alternative but related models for the plasma membrane binding of the K-ras4B carboxy-terminal sequence. First, the 'target locus' for this sequence may comprise simply the lipid bilayer of the plasma membrane. In this case, the preferential localization of this sequence to the plasma membrane could be explained by postulating that the K-ras4B carboxy terminus, which discriminates very sensitively between bilayers of differing surface charge (*34, 35*), senses a significantly higher surface potential, and therefore binds with substantially greater affinity, at the cytoplasmic surface of the plasma membrane (or particular regions within it) than at the cytoplasmic surfaces of other cellular membranes. In support of the feasibility of this proposal, we note that the plasma membrane (in contrast to at least some intracellular membranes) exhibits a strongly asymmetric distribution of anionic phopholipids in favor of its cytoplasmic surface [reviewed in (*37*)] and moreover may exhibit lateral concentration of certain negatively charged lipids (e.g., phosphoinositides) in particular submembrane domains (*38, 39*). An alternative possibility is that the target locus for the K-ras4B targeting sequence at the plasma membrane comprises both lipid and protein components. In this latter case, however, from our present results one would have to conclude that the protein component imposes virtually no restrictions on the possible structures of a bound targeting sequence but simply creates a diffuse and quite extensive region of enhanced local (negative) charge density. Either of these proposals provides a natural explanation for the high net positive charge (+8) of the K-ras4B targeting sequence, which given the presence of the methylated/farnesylated cysteine residue is not essential to confer simple membrane binding within the cell (*11, 40*) but which is required to provide a high degree of discrimination among membrane environments of differing surface charge density (*31, 34, 35*).

Two additional findings from the present study aid in defining better the mechanism(s) by which K-ras4B is recruited to the plasma membrane. First, the results of our in vitro assays suggest that the plasma membrane binding of the EGFP/K-ras4B(171−188) chimera is of high affinity yet reversible, as Yokoe and Meyer (*41*) also concluded in their study of a GFP/K-ras4B chimera in intact RBL cells. A similar study (*42*) concluded that in intact COS-7 cells the same chimera does not dissociate from the plasma membrane on a time scale of several seconds but left open the possibility that dissociation could occur on somewhat longer time scales, as we in fact observe in vitro using the EK chimera. The reversibility of plasma membrane binding observed for these chimeric proteins implies that the mechanism(s) restoring them to the plasma membrane after dissociation must be relatively rapid, given the high degree of plasma membrane association observed for such proteins in steady-state [this study and (*5*−*7, 11, 12*)].

Recent evidence has shown that prenylated but incompletely processed forms of K-ras4B associate with intracellular membranes (*11, 12*) and microtubules (*18, 19*), in a strictly prenylation-dependent manner, prior to recruitment of the fully processed protein to the plasma membrane. To date, however, the mechanistic relationship between this intracellular routing of nascent K-ras4B and the ultimate delivery of the mature protein to the plasma membrane has remained unclear. It is thus interesting to observe that EGFP chimeras bearing variants of the K-ras4B targeting sequence can be efficiently and specifically delivered to the plasma membrane even when they lack a prenyl moiety (or indeed any lipidic residue). This finding suggests that the specific (prenylation-dependent) subcellular trajectory followed by the wild-type K-ras4B targeting sequence during processing, while it clearly ensures the proper maturation of the protein (*11, 43, 44*), and possibly mediates important intracellular signaling or other functions of K-ras4B (*11, 19*), may not

[3] From our findings, we cannot rule out the possibility that full-length K-ras4B may exhibit interactions with intracellular components that chimeric proteins bearing the K-ras4B carboxy terminus alone do not, and that as a result of such interactions K-ras4B may reach the plasma membrane using a pathway or mechanism that differs at least in detail from that exhibited by the chimeric species examined here. However, we note that the extensive investigations reported by Choy et al. (*11*) did not reveal any substantive differences between the intracellular trafficking and ultimate plasma membrane delivery of K-ras4B itself and that observed for chimeric proteins bearing the K-ras4B targeting sequence.

also directly mediate the transport of the mature protein to the plasma membrane itself. Our results [and the previous findings of Choy et al. (*11*)] are in fact consistent with the possibility that proteins bearing the K-ras4B targeting sequence may ultimately reach the plasma membrane by simple diffusion. However, further study will be required to assess this possibility more rigorously.[3]

The present study has focused on the plasma membrane-targeting role of the carboxy-terminal region of K-ras4B. However, it must be noted that the farnesylated targeting sequences of the ras proteins also appear to influence ras protein function through effects that extend beyond localizing these proteins to the plasma membrane (*7*, *45*−*49*). In principle, such further influences of the carboxy-terminal targeting sequences on ras protein function could reflect direct interactions of these sequences with cellular effector and/or modulatory proteins, modulation of the distributions of the different ras proteins between distinct domains within the plasma membrane (*50*), or some combination of these effects. Further study will be required to elucidate (and differentiate) these additional contributions of the carboxy-terminal hypervariable sequences to the normal and oncogenic functions of K-ras4B and other ras family members.

ACKNOWLEDGMENT

We thank Carol Miyamoto and Drs. Rhoda Blostein, Jerry Pelletier (McGill University), and Ken Jacobson (Chapel Hill) for useful discussions, and the laboratory of Dr. Danny Baranes (Anatomy, McGill University) for assisting us in the use of the confocal-microscope facility.

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BI000512Q