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Biochemistry

Polylysine domain of K-ras 4B protein is crucial for malignant transformation

(isoprenylation/Ki-Ras/membrane association/transforming activity/carboxyl-terminal processing)

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ABSTRACT **Previous studies have shown that posttranslational modifications are required for both oncogenic K-ras 4B protein membrane binding and transforming activity. In addition, Hancock *et al.* [Hancock, J. F., Patterson, H. & Marshall, C. J. (1990) *Cell* 63, 133–139] found that a polylysine domain contained at the C terminus of K-ras 4B was also absolutely essential for K-ras 4B membrane binding but, surprisingly, neither the polylysine domain nor membrane binding was required for transforming activity. We have performed similar studies, but our results are distinctly different. Our studies indicate that the polylysine domain is crucial for K-ras 4B transforming activity. Moreover, we demonstrate that although the polylysine domain increases K-ras 4B membrane binding, significant amounts of membrane binding can occur in the absence of this domain. Finally, while our studies are consistent with the notion that membrane binding is required for K-ras 4B transforming activity, we show that membrane binding, in and of itself, is not sufficient for efficient K-ras 4B transforming activity.**

Ras proteins are critical components of signal transduction pathways that regulate cell growth and differentiation. Recent studies have shown that extracellular signals (growth factors, antigens, interleukins, trophic factors, etc.) can stimulate cell surface receptors to become coupled [via Src homolog 2/3 (SH2/SH3)-containing adaptor proteins, such as Grb2] to guanine nucleotide exchange factors (such as Sos), which can in turn stimulate the conversion of inactive Ras·GDP to active Ras·GTP (1–3). Ras·GTP can subsequently interact with the serine/threonine kinase Raf-1, and following activation, Raf-1 can initiate a cascade of phosphorylation [including MAP kinase kinase (MEK), mitogen-activated protein kinase (MAPK), and pp90 Rsk] that ultimately phosphorylates nuclear transcription factors (such as Fos and Jun) and activates gene expression (1–3).

Human cells contain four distinct but strikingly homologous Ras proteins (H-ras, N-ras, K-ras 4A, and K-ras 4B) which primarily differ only in their last 25 aa. [K-ras 4A and K-ras 4B are encoded by the same gene (*KRAS*) but contain alternative fourth exons. In human cells K-ras 4B is transcribed 10- to 20-fold more than K-ras 4A (4–6).] Although each of these Ras proteins can be converted into oncogenic proteins by amino acid substitutions at codon 12, 13, or 61, oncogenic K-ras proteins are most frequently detected in human malignancies (4).

Oncogenic Ras proteins are synthesized in the cytosol as inactive precursors and subsequently undergo a series of posttranslational modifications which enable them to bind to the inner surface of cell plasma membranes and transform cells (7–12). These posttranslational modifications are signaled by the C-terminal 4 aa of Ras proteins and include isoprenylation, truncation, methylation, and palmitoylation (8–17). Each of the Ras proteins undergoes isoprenylation, truncation, and methylation. However, only H-ras, N-ras, and K-ras 4A proteins undergo palmitoylation (8, 9). Palmitoylation, as well as isoprenylation, methylation, and truncation, is important for H-ras, N-ras, and K-ras 4A membrane binding (8). However, membrane binding of nonpalmitoylated K-ras 4B is equivalent to that of H-ras, N-ras, and K-ras 4A (8, 9, 12). Instead of undergoing palmitoylation, K-ras 4B (but not H-ras, N-ras, or K-ras 4A) contains a polylysine domain at its C terminus; and a recent paper (18) has indicated that this polylysine domain is crucial for K-ras 4B membrane binding. It has been inferred, therefore, that two signals—posttranslational modifications and a polylysine domain—are required for K-ras 4B membrane binding. Further, the notion that membrane binding is essential for Ras transforming activity has been questioned, because the aforementioned study demonstrated that the polylysine domain, although essential for K-ras 4B membrane binding, was not required for K-ras 4B transforming activity. We have performed similar studies, but our results are distinctly different. The studies reported herein demonstrate that the polylysine domain is crucial for K-ras 4B transforming activity and increases, but is not absolutely essential for, K-ras 4B membrane binding. Moreover, we show that these effects are mediated through ionic interactions. Finally, although our results are consistent with the premise that membrane binding is required for K-ras 4B transforming activity, they indicate that membrane binding, in and of itself, is not sufficient for complete K-ras 4B transforming activity.

MATERIALS AND METHODS

Construction of K-ras 4B and GTPase-activating Protein (GAP)/K-ras 4B Mutants. Oligonucleotide-directed mutagenesis, PCR mutagenesis, and construction of DNA vectors were performed as described (12, 19). For K-ras 4B mutants, lysine (AAA or AAG codon) was changed to glutamine (CAA or CAG) or arginine (AGA or AGG). To facilitate distinction of mutant versus endogenous K-ras 4B by SDS/PAGE, mutations were introduced into a transforming ($Gly^{12} \rightarrow Val$) human K-ras 4B cDNA (6K) that encodes a non-Ras 10-aa N-terminal leader (20). GAP/6K/CAAX (in the CAAX sequence, C is cysteine, A represents an aliphatic residue, and X can be any of several amino acids) and GAP/6Q/CAAX hybrids, containing the catalytic domain (aa 705–1047) of full-length Ras p120 GAP at their N termini and the C-terminal 18 aa of 6K or 6Q at their C termini, were constructed by using a human GAP cDNA (21). For GAP/6K/SAAX and GAP/6Q/SAAX, the cysteine (TGT) of 6K or 6Q was changed to serine (AGT). Tables 1 and 2 summarize the

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Abbreviation: GAP, GTPase-activating protein.

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Table 1. Biochemical and biological properties of K-ras 4B proteins

Protein	Amino acid sequence*	Membrane association,† %	Isoprenylation‡	Methylation,§ alkali released/alkali-stable ^{3}H cpm	Transforming activity,¶ % NIH 3T3	Transforming activity,¶ % Rat-1
6K‖	RKHKEKMSKDGKKKKKKSKTKVIM	92	+	872/4116	100	100
6R	----------RRRRRR--------	89	+	505/1945	108	117
2Q	----------Q-Q--------	83	+	1229/3241	11	<2
4Q	----------QQ-Q-Q--------	71	+	775/2911	8	<2
6Q	----------QQQQQQ--------	52	+	996/2324	3	<1
SVIM**	----------S---	<5	−	58/2878	0	0
C**	----------C	<5	−	40/1567	0	0

*The site and nature of amino acid substitutions are indicated. Hyphens indicate sites where amino acid sequences are identical to 6K sequences.
†Radioactivity in K-ras 4B proteins immunoprecipitated from membrane (cpm_m) and cytosol (cpm_c) fractions of [^{35}S]methionine/cysteine-labeled cells was quantitated by liquid scintillation spectroscopy; % membrane association = $[cpm_m/(cpm_m + cpm_c)] \times 100$.
‡Isoprenylation of K-ras 4B protein was assessed as outlined in the legend to Fig. 3.
§K-ras 4B proteins immunoprecipitated from [*methyl*-^{3}H]methionine-labeled cells were treated with 1 M NaOH, and methylation was assessed by comparing the amount of ^{3}H released from K-ras 4B ([^{3}H]methyl groups are released as [^{3}H]methanol) with the amount of ^{3}H stably incorporated into K-ras 4B (as [^{3}H]methionine residues).
¶NIH 3T3 and Rat-1 cells were transfected with 10 ng and 1 μg, respectively, of K-ras 4B plasmid DNA, and morphologically transformed foci were quantitated after 14 and 21 days, respectively. Results represent transforming activity relative to the transforming activity of 6K. NIH 3T3 and Rat-1 cells typically develop approximately 3800 and 280 foci, respectively, per μg of 6K DNA.
‖K-ras 4B protein containing an intact polylysine domain and an activating Val^{12} mutation.
**Previously described K-ras 4B mutants which are not isoprenylated, methylated, or truncated (12).

specific K-ras 4B and GAP/K-ras 4B mutants, respectively. Nucleotide sequences of mutants were verified by dideoxynucleotide chain-termination sequencing (22), and mutants were subsequently cloned into the pZIP-Neo-SV(X)1 retrovirus expression vector (23).

Cell Transfection and Transformation Assays. For K-ras 4B constructs, NIH 3T3 cells and Rat-1 cells were transfected, by calcium phosphate precipitation, with 10 ng and 1 μg, respectively, of pZIP K-ras 4B plasmid DNA (12). For GAP/K-ras 4B constructs, NIH 3T3 cells were cotransfected with 20 ng of pZIP GAP/K-ras 4B plasmid DNA and 1 μg of pMUT-1 (pMUT-1 contains a full-length, transforming, genomic H-*ras* sequence, containing an activating $Gln^{61} \rightarrow$ Leu mutation; ref. 24). Cotransfection with transforming H-*ras* was necessary, because GAP/K-ras 4B hybrid proteins were growth inhibitory to nontransformed NIH 3T3 cells. For focus-forming transformation assays, transfected NIH 3T3 and Rat-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/10% calf serum and DMEM/Ham's F10 medium (1:1)/10% calf serum, respectively. Transformed foci in NIH 3T3 cells and Rat-1 cells were quantified after 14 and 21 days, respectively (12).

Subcellular Localization of K-ras 4B and GAP/K-ras 4B Proteins. *Subcellular fractionation of proteins.* NIH 3T3 cells transfected with K-ras 4B or GAP/K-ras 4B were selected with G418, labeled overnight with [^{35}S]methionine/cysteine (200 μCi/ml; 1 μCi = 37 kBq), and separated into crude membrane and cytosol fractions; K-ras 4B or GAP/K-ras 4B proteins contained in these fractions were immunoprecipitated with anti-Ras monoclonal antibody Y13-259 (25) or anti-GAP monoclonal antibody G73 (kindly provided by Frank McCormick, Onyx Corporation), respectively, and analyzed by SDS/PAGE and fluorography (12). To quantify the protein contained in cytosol and membrane fractions, proteins were eluted from gels, and incorporated radioactivity was quantified by liquid scintillation spectrometry (26).

Immunofluorescence of K-ras 4B proteins. G418-selected cells expressing K-ras 4B were grown on glass coverslips, fixed in 2% formaldehyde, permeabilized in 0.2% saponin, and incubated successively with primary anti-Ras antibody (Y13-238), biotinylated goat anti-rat IgG secondary antibody, and fluorescein-conjugated streptavidin. Immunofluorescence was visualized on a confocal fluorescence microscope. Y13-238 antibody does not efficiently detect endogenous Ras proteins of NIH 3T3 cells (25).

Detection of Posttranslational Modifications of K-ras 4B and GAP/K-ras 4B Proteins. *Detection of isoprenylation.* Isoprenylation was assessed by determining whether K-ras 4B or GAP/K-ras 4B proteins incorporated label derived from [^{3}H]mevalonic acid (12). G418-selected cells were labeled overnight with (*R,S*)-[^{3}H]mevalonolactone (100 μCi/ml, in the presence of 50 μM compactin; ref. 27), and K-ras 4B proteins were immunoprecipitated and analyzed by SDS/PAGE and fluorography.

Detection of methylation. Methylation was assessed by comparing ^{3}H cpm released with ^{3}H cpm stably retained following alkali treatment of Ras proteins that had been metabolically labeled with [*methyl*-^{3}H]methionine [the radioactivity from ^{3}H is incorporated into methionine residues and methyl groups of proteins (13), but only the methyl groups are alkali labile]. Briefly, G418-selected cells were labeled overnight with [*methyl*-^{3}H]methionine (200 μCi/ml; NEN), and proteins were immunoprecipitated and analyzed by SDS/PAGE and fluorography (12). Dried gel sections containing K-ras 4B or GAP/K-ras 4B proteins were cut out and treated with 1 M NaOH, and radioactivity released from proteins (as [^{3}H]methanol) or stably retained by proteins (as [^{3}H]methionine) was quantitated by liquid scintillation spectrometry (13).

Table 2. Biochemical properties of GAP/K-ras 4B hybrid proteins

Protein	Amino acid sequence	Membrane association, %	Isoprenylation	Methylation, alkali-released/alkali-stable ^{3}H cpm
GAP/6K/CAAX*	(GAP) SKDGKKKKKKSKTKCVIM	81	+	392/1340
GAP/6Q/CAAX	(GAP) -----QQQQQQ--------	51	+	370/1631
GAP/6K/SAAX	(GAP) ----------S---	25	—	34/991
GAP/6Q/SAAX	(GAP) -----QQQQQQ-----S---	28	—	46/1439

*Hybrid protein containing the catalytic domain (aa 705–1047) of full-length Ras p120 GAP at its N terminus and the C-terminal 18 aa of 6K at its C terminus.

RESULTS

Membrane Association of K-ras 4B Mutants. To examine the role of the polylysine domain in K-ras 4B membrane binding, we transfected NIH 3T3 cells with K-ras 4B constructs encoding substitutions of neutral glutamine or basic arginine for the six contiguous basic lysine residues (Table 1) and determined the subcellular location of the encoded mutants. The vast majority of 6K and 6R, containing six contiguous lysines or arginines, respectively, was associated with the membrane fraction (92% and 89%, respectively; Fig. 1). In contrast, substitution of two (2Q), four (4Q), or six (6Q) glutamines for the corresponding lysines caused a gradual (but incomplete) decrease in the amount of Ras protein associated with the membrane fraction. (The differing mobilities of the K-ras 4B proteins correlate with their differing net charges.) To demonstrate that the plasma membrane is the specific membrane component with which K-ras 4B associates, the subcellular location of K-ras 4B was also evaluated with confocal immunofluorescence microscopy. The 6K, 6R, 2Q, and 4Q proteins were predominantly associated with the plasma membrane, while substantial amounts of 6Q are associated with the cytoplasm, as well as the plasma membrane (Fig. 2). These results suggest that although the six contiguous lysines increase K-ras 4B membrane binding, substantial amounts of membrane binding can occur in their absence. Moreover, since membrane association of 6K and 6R are equivalent, these results indicate that the polylysine domain increases K-ras 4B membrane binding as a result of its positive charge.

Posttranslational Modifications of K-ras 4B Mutants. Since posttranslational modifications are crucial for K-ras 4B membrane binding, and 2Q, 4Q, and 6Q have progressively decreased amounts of membrane binding, we determined whether these mutants might have comparably decreased levels of posttranslational modifications. Fig. 3 and Table 1 demonstrate that 6K, 6R, 2Q, 4Q, and 6Q are all efficiently isoprenylated and methylated. (The amount of labeling with [^{3}H]mevalonolactone relative to the amount of labeling with [^{35}S]methionine/cysteine was comparable for all the K-ras 4B proteins; data not shown.) These results demonstrate that the polylysine domain is not required for CAAX-signaled posttranslational modifications to occur and suggest that the polylysine domain directly increases K-ras 4B membrane binding. These results also indicate that posttranslational modifications, although absolutely required, are not in themselves completely sufficient for full K-ras 4B membrane binding.

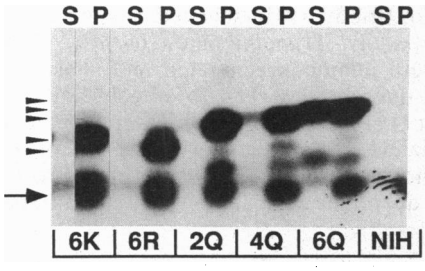

FIG. 1. Subcellular fractionation of K-ras 4B proteins. G418-selected NIH 3T3 cells were labeled with [^{35}S]methionine/cysteine and separated into crude membrane (pellet, P) and cytosol (supernatant, S) fractions, and K-ras 4B proteins contained in these fractions were immunoprecipitated and analyzed by SDS/PAGE and fluorography. NIH, untransfected NIH 3T3 cells; arrowheads, positions of mutant K-ras 4B proteins containing a 10-aa N-terminal leader; solid arrow, position of endogenous Ras proteins. The differing mobilities of K-ras 4B proteins correlate with their differing net charges.

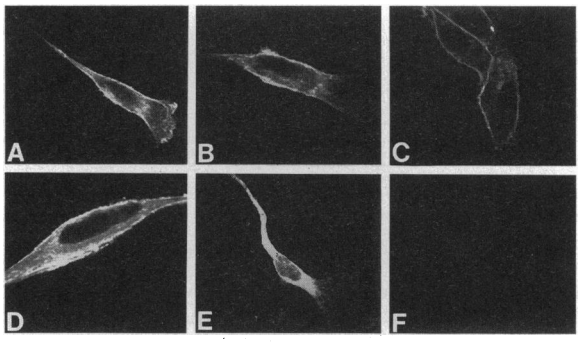

FIG. 2. Immunofluorescence of K-ras 4B proteins in cells expressing 6K (*A*), 6R (*B*), 2Q (*C*), 4Q (*D*), or 6Q (*E*). Control NIH 3T3 cells (*F*) contained no exogenous K-ras 4B. G418-selected NIH 3T3 cells were fixed in formaldehyde and permeabilized with saponin, and K-ras 4B proteins contained in these cells were stained with fluorescein. Immunofluorescence was visualized on a confocal fluorescence microscope.

Effect of the Polylysine Domain and CAAX Motif on Subcellular Location of a Heterologous, Cytoplasmic GAP. To further assess the relative contributions of the polylysine domain and/or posttranslational modifications to K-ras 4B membrane binding, we determined whether the addition of a polylysine domain and/or posttranslational modifications to the C terminus of the catalytic domain of Ras p120 GAP (a cytoplasmic protein which stimulates Ras GTPase activity; ref. 21) could convert the predominantly cytoplasmic catalytic GAP protein into a membrane-bound protein. Table 2 summarizes the specific GAP/K-ras 4B hybrid proteins generated. GAP/6K/CAAX, containing six contiguous lysines, was predominately membrane-associated, whereas GAP/6Q/CAAX containing six glutamines substituted for the lysines, was only ≈50% membrane associated (Fig. 4). (Both GAP/6K/CAAX and GAP/6Q/CAAX are isoprenylated and methylated; Table 2 and data not shown.) Moreover, when isoprenylation, methylation, and truncation were prevented (GAP/6K/SAAX and GAP/6Q/SAAX), only ≈25% of the hybrid proteins were membrane associated. (Catalytic GAP, containing no K-ras 4B sequences, was also ≈25% membrane associated; data not shown.) Our results with these GAP/K-ras 4B hybrids parallel our results with full-length K-ras 4B proteins. Although the polylysine domain (in conjunction with posttranslational modifications) increases membrane binding of catalytic GAP, substantial amounts of membrane binding occur with posttranslational modifications alone, in the absence of the polylysine domain. Our results also

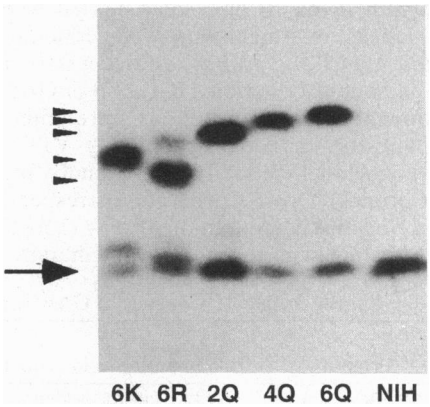

FIG. 3. Isoprenylation of K-ras 4B proteins. G418-selected NIH 3T3 cells were labeled with [^{3}H]mevalonolactone, and K-ras 4B proteins contained in these cells were immunoprecipitated and analyzed by SDS/PAGE. Arrowheads, mutant K-ras 4B proteins; solid arrow, endogenous Ras proteins.

suggest that the C-terminal 18 aa of K-ras 4B (in conjunction with posttranslational modifications) may be sufficient for K-ras 4B membrane binding.

Transformation Efficiency of K-ras 4B Mutants. Given the role of the polylysine domain in K-ras 4B membrane binding, we determined its role in K-ras 4B transforming activity. As shown in Table 1, 6K and 6R are highly and equally transforming. In contrast, 2Q, 4Q, and 6Q all have drastically reduced transforming activity. Moreover, there is little correlation between the amount of membrane association and the transforming activity of mutant K-ras 4B proteins. For instance, in NIH 3T3 cells, the transforming activity of 2Q is decreased 90%, but its membrane association is decreased only 10%, relative to 6K. Similarly, 4Q and 6Q have 92% and 97%, respectively, decreases in transforming activity but only 23% and 44%, respectively, decreases in membrane association. These results suggest that a completely intact polylysine domain may be required for optimal K-ras 4B transforming activity. In addition, these results demonstrate that membrane binding, in and of itself, is not sufficient for efficient K-ras 4B transforming activity.

DISCUSSION

Our results indicate that the polylysine domain is crucial for K-ras 4B transforming activity. Indeed, the almost equivalent decrease in transforming activity of K-ras 4B mutants containing two, four, or six Lys → Gln substitutions (2Q, 4Q, and 6Q) suggests that all six lysines of the polylysine domain may be required for efficient transforming activity. In addition, although the polylysine domain significantly increases K-ras 4B membrane binding, ≈50% of K-ras 4B remains membrane bound in the absence of this domain. Finally, our studies (and the studies of Hancock *et al.*; ref. 28), which demonstrate that positively charged arginine residues can very efficiently substitute for the six lysines of K-ras 4B, suggest that the polylysine domain likely facilitates membrane binding and enables cell transformation via ionic interactions with a negatively charged domain of a target protein and/or negatively charged membrane phospholipid headgroups.

The abrupt and dramatic decrease in transforming activity of 2Q, 4Q, and 6Q K-ras 4B mutants—despite their gradual and modest decrease in membrane association—suggests that membrane binding, in and of itself, is not sufficient for efficient K-ras 4B cell transformation. Binding to a specific membrane target site (and/or lipid environment) may be equally or more important than the absolute amount of membrane binding. Specific membrane binding can occur through a variety of noncovalent bonds. Therefore, the polylysine domain (through its ability to form ionic bonds) and posttranslational modifications (through their ability to facilitate hydrophobic and van der Waals interactions) could create a binding domain in K-ras 4B that specifically interacts with its membrane target site. Accordingly, although two Lys → Gln substitutions may not significantly decrease absolute membrane binding, these substitutions could structurally alter the K-ras 4B binding domain and thereby prevent specific membrane binding, signal transduction, and cell transformation. [Since previous studies have shown that an N-terminal myristate (29, 30) or a C-terminal geranylgeranyl group (28, 31) can efficiently substitute for the farnesyl group of oncogenic, but not normal, Ras proteins, a nonspecific lipid membrane anchor, in conjunction with a polylysine domain, may be sufficient for oncogenic, but not normal, Ras proteins.] Alternatively, the polylysine domain may facilitate the binding of K-ras 4B to a critical nonmembrane target. At any rate, whether or not the polylysine domain (alone or in conjunction with posttranslational modifications) enables K-ras 4B to bind a membrane and/or nonmembrane target(s)—if this binding is required for transforming activity—mutations which prevent this binding will prevent transforming activity.

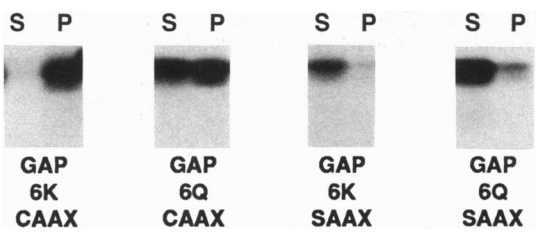

FIG. 4. Subcellular fractionation of GAP/K-ras 4B proteins. G418-selected NIH 3T3 cells were labeled with [^{35}S]methionine/cysteine and separated into crude membrane (pellet, P) and cytosol (supernatant, S) fractions, and GAP/K-ras 4B proteins contained in these fractions were immunoprecipitated and analyzed by SDS/PAGE and fluorography.

As noted above, ≈50% of K-ras 4B remains membrane associated in the absence of a polylysine domain. Posttranslational modifications could account for this residual membrane association. Alternatively, since 16 of the C-terminal 25 aa of K-ras 4B are charged (see Table 1), some or all of these additional charged residues could also account for the residual membrane association.

Our results clearly indicate that posttranslational modifications, in the absence of a polylysine domain, are not sufficient for complete membrane binding or efficient transforming activity. Likewise, previous studies have shown that the polylysine domain, in the absence of posttranslational modifications, is also not sufficient for membrane binding or transforming activity (see Table 1, SVIM and C mutants; refs. 8, 12, 32, and 33). However, the polylysine domain, in conjunction with posttranslational modifications, is sufficient to promote membrane binding of the cytosolic catalytic domain of Ras p120 GAP as well as cytosolic protein A (28), and this membrane-bound catalytic GAP (but not cytosolic catalytic GAP) can inhibit the transforming activity of oncogenic H-ras (ref. 34 and unpublished results). Polylysine domains and posttranslational modifications could, therefore, represent a general cellular mechanism to facilitate the membrane binding and biologic activity of a variety of different proteins.

We do not know whether there are significant biologic differences between K-ras 4B, which contains a polylysine domain, and H-ras, N-ras, and K-ras 4A proteins, which are palmitoylated. However, other Ras superfamily proteins (such as Rap and Rho) also have palmitoylated and nonpalmitoylated counterparts; and the nonpalmitoylated counterparts often contain polybasic domains (35–37). Since the polylysine domain should form ionic bonds, whereas palmitate should facilitate hydrophobic and van der Waals interactions, the polylysine domain and palmitate modification(s) may direct K-ras 4B and H-ras, N-ras, or K-ras 4A proteins, respectively, to different targets. Furthermore, since palmitoylation is transient ($t_{1/2}$ < 20 min; ref. 36), there could be significant differences in the way proteins containing permanent polybasic domains versus transient palmitate modifications are regulated.

The studies presented here were performed concurrently with those of Hancock *et al.* (18, 28). However, there are several distinct differences between our results. They observed a ≥20-fold decrease in membrane association but found only an ≈2-fold reduction in transforming activity when glutamines were substituted for the six contiguous lysines of [Val^{12}]K-ras 4B. In contrast, we found a <2-fold reduction in membrane association and a 30-fold decrease in transforming activity with our similarly substituted [Val^{12}]K-ras 4B mutant. The implications of our studies are quite different from those of the studies by Hancock *et al.* Their

results suggest that (*i*) two signals—posttranslational modifications and a polylysine domain—are absolutely essential for K-ras 4B membrane binding and (*ii*) neither the polylysine domain nor membrane binding is required for K-ras 4B transformation. In contrast, our results indicate that the polylysine domain is crucial for transformation and demonstrate that significant amounts of membrane binding can occur in the absence of the polylysine domain. While our studies are consistent with the notion that membrane binding is required for K-ras 4B transforming activity, our results clearly indicate that membrane binding, in and of itself, is not sufficient for efficient transforming activity. The difference in transforming activity observed by us and by Hancock *et al.* is most likely explained by the different susceptibilities of our two clones of NIH 3T3 cells to transformation, because we do not detect a significant difference in transforming activity between their or our Gln_6-substituted mutant when we transfect these constructs into our NIH 3T3 or Rat-1 cells (<1% and 3%, or <1% and <1%, transforming activity, respectively, relative to [Val^{12}]K-ras 4B). In regard to the observed differences in membrane association, we utilized NIH 3T3 cells both to quantitate transforming activity and to perform subcellular fractionations, whereas Hancock *et al.* utilized NIH 3T3 cells to quantitate transforming activity but performed subcellular fractionations on COS cells. When we performed subcellular fractionations on NIH 3T3 cells transfected with either their or our Gln_6-substituted K-ras 4B mutant, we found that the levels of membrane association of these two proteins were not significantly different (45% and 51% membrane associated, respectively).

We dedicate this paper to the late Chao-Yu Kuo. Without her superb technical assistance, this work would not have been possible. In addition, we gratefully acknowledge the excellent technical assistance of Jeffrey R. Bourne, Maja Vollenweider, and Yan Jin; George Klier, Richard Adams, and Helen Plutner for their assistance with immunofluorescence assays; John Hancock and Chris Marshall for their Gln_6-substituted K-ras 4B construct and useful discussions; Frank McCormick for GAP cDNA and GAP antibodies; Michael Klemsz for helpful advice; and Monica Bartlett for excellent secretarial assistance. This work was supported by a National Institutes of Health grant to J.H.J. (CA54298) and by grants from the Tobacco-Related Disease Research Program of the University of California to J.H.J. (RT 298, KT 120).

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