Lipid-Binding Characteristics of the Polybasic Carboxy-Terminal Sequence of K-*ras*4B†

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Received December 15, 1997; Revised Manuscript Received March 3, 1998

ABSTRACT: We have examined the association with lipid vesicles of fluorescent lipidated peptides based on the farnesylated, polybasic carboxy-terminal region of mature K-*ras*4B, which functions physiologically as an autonomous plasma membrane-targeting motif. While the peptides bind to neutral lipid (phosphatidylcholine/phosphatidylethanolamine) vesicles with relatively low affinity, the vesicle-binding affinity increases exponentially as increasing amounts of anionic lipids are incorporated into the vesicle bilayers. Competitive vesicle-binding experiments reveal that the K-*ras*4B carboxy-terminal sequence accordingly discriminates strongly between lipid surfaces of differing surface charge, such that two lipid bilayers differing in anionic lipid content by 10 mol % will show a 45-fold preferential accumulation of the lipidated peptide in the more negatively charged surface. At the same time, the carboxyl-terminal region of K-*ras*4B exhibits no preferential binding to particular anionic lipids, including the polyanionic species phosphatidylinositol-4′-phosphate and phosphatidylinositol-4′,5′-bisphosphate, beyond that predicted on the basis of surface-charge effects. The K-*ras*4B carboxyl-terminal sequence dissociates rapidly (with half-times of seconds or less) from lipid bilayers containing up to 40 mol % anionic lipid. These results suggest that the targeting of the mature K-*ras*4B carboxy-terminus to the plasma membrane, if it is based on interactions with plasma membrane lipids, is not mediated by a kinetic-trapping mechanism or by specific binding to particular anionic lipids but may rest on the sensitive surface potential-sensing function of this region of the protein.

The protooncogenic proteins H-, K-, and N-*ras* are found primarily associated with the plasma membrane in their mature form, and this targeting appears to be important to the biological activities of both the normal and the oncogenically activated forms of these proteins (*1*−*8*). The extreme carboxy-terminal sequences of H-*ras* and K-*ras*4B, when processed to terminate in an *S*-farnesyl/*O*-methylcysteine residue, are sufficient to target both these and chimeric proteins to the plasma membrane (*1*−*12*). In K-*ras*4B the essential targeting region comprises a polybasic sequence immediately preceding the terminal farnesylated/methylated cysteine residue, while for H- and N-*ras* and K-*ras*4A the modified cysteine is preceded instead by a short sequence containing one or more palmitoylation sites.

The mechanism by which the polybasic/farnesylated carboxy-terminal domain of mature K-*ras*4B targets the protein to the plasma membrane remains unclear. While K-*ras*4B could in principle be targeted to the plasma membrane by association with a proteinaceous receptor, no candidate receptor has yet been identified that recognizes specifically the polybasic carboxy-terminal targeting sequence of the mature protein. Mutational analysis has demonstrated that the farnesyl group of K-*ras*4B can be replaced with an N-terminal myristoyl group without impairing plasma membrane targeting and has suggested that the overall polybasic character of the carboxy-terminal region, rather than its precise amino acid sequence, may be central to its plasma membrane-targeting function (*2*, *3*, *5*). Finally, K-*ras*4B and chimeric proteins bearing the K-*ras*4B carboxyl-terminal sequence are targeted to the plasma membrane even when expressed at levels much higher than the endogenous protein (*2*−*4*). These observations have led to suggestions that the carboxy-terminal sequence of mature K-*ras*4B may interact with plasma membrane lipids rather than with a proteinaceous receptor (*2*, *3*).

The hypothesis that the carboxyl-terminal region of K-*ras*4B may interact with membrane lipids raises the question of how such interactions could promote a preferential association of the protein with the plasma membrane. Three possible mechanisms to achieve such a localization by a lipid-based mechanism can be envisioned, as follows:

(1) After completion of processing and initial delivery to the plasma membrane [processes in which microtubules may play a role (13)], the carboxy-terminal region of K-*ras*4B could potentially exhibit a very slow rate of *spontaneous* desorption from the plasma membrane lipid bilayer. In this case, the plasma membrane localization of the mature protein could subsequently be maintained "kinetically" by factors regulating its membrane association/dissociation, as observed for example for the *rab* GTPases (*14*).

(2) The carboxy-terminal region of mature K-*ras*4B could associate with high affinity with specific anionic lipids enriched in the plasma membrane.

(3) The carboxyl-terminal domain of the mature protein could bind reversibly but preferentially to the plasma membrane through simple electrostatic forces, if the cyto-

† This work was supported by a grant from the Medical Research Council of Canada.

S0006-2960(97)03077-8 CCC: $15.00 © 1998 American Chemical Society
Published on Web 05/02/1998

plasmic face of this membrane exhibits a significantly more negative surface charge than other cellular membranes.

In this study we have used fluorescent lipidated peptides, based on the polybasic carboxyl-terminal domain of K-*ras*4B, to evaluate the feasibility of each of the above mechanisms as a basis for the preferential association of this region of the mature protein with the plasma membrane. Our results do not provide support for either of the first two "lipid hypotheses" presented above. However, our data indicate that the polybasic carboxyl-terminal sequence of K-*ras*4B exhibits a high ability to discriminate between lipid bilayers of different surface charge, a property that could play a role in the plasma membrane-targeting function of this region of the protein.

MATERIALS AND METHODS

Materials. PIP[1] and PIP_2 were obtained from Sigma (St. Louis, MO); all other phospholipids were purchased from Avanti Polar Lipids (Alabaster, AL). Peptides were synthesized as described below using protected amino acids and other reagents from Novabiochem (San Diego, CA). Intermediate protected peptides in solution syntheses were purified by flash chromatography on silica gel 60 (*15*), eluting with appropriate gradients (typically 0−3%) of methanol in methylene chloride. Peptides were finally purified by high-pressure liquid chromatography on a C18/C2 reverse-phase column (Super-Pac Prep-S, Pharmacia, Point-Claire, Québec), eluting with a gradient of 0−50% acetonitrile in water; the masses of the purified peptides were verified by electrospray-ionization mass spectrometry.

Peptides Bimta-KTKC(X)-OMe, where X = undecyl or farnesyl, were synthesized by solution synthesis using Boc chemistry (*16*), starting from *S-tert*-butylmercaptocysteine methyl ester and using Fmoc protection for the lysine side chains. *S*-Bimanylmercaptoacetic acid (*17*) was finally coupled to the amino terminus of the side-chain-protected tetrapeptide, and the cysteine sulfhydryl group was then deprotected with 1,4-butanedithiol (*18*) and coupled to either farnesyl or *n*-undecyl bromide in the presence of $KF{\cdot}2H_2O$ (*19*, *20*). After purification by preparative TLC on silica gel 60, the S-alkylated peptides were finally N^{ϵ}-deprotected with 20% piperidine in dimethylformamide at 0 °C for 30 min and purified by preparative TLC, developing with 50/15/5/5/2 (v/v/v/v/v) CH_2Cl_2/acetone/acetic acid/methanol/water.

Peptides Bimta-GK_xSKTKC(undecyl)-OMe were synthesized by a fragment-condensation approach. Protected peptides Bimta-G(K[Boc])$_x$S-OH were prepared by solid-phase synthesis using Fmoc chemistry, coupling *S*-bimanylmercaptoacetic acid as the N-terminal residue. The protected peptide FmocK(Boc)T(tBu)K(Boc)C(StBu)-OMe was prepared by solution synthesis using Fmoc chemistry and then S-deprotected and coupled to undecyl bromide as described above. After purification of the protected tetrapeptide by column chromatography the Fmoc group was removed with dimethylformamide/piperidine, and the peptide was coupled to protected fragments Bimta-G(K[Boc])$_x$S−OH at 0 °C in the presence of benzotriazol-1-yloxy-(trispyrrolidino)phosphonium hexafluorophosphate, 1-hydroxybenzotriazole and diisopropylethylamine (1.5, 4, and 3 molar equiv, respectively). The protected product was purified by TLC on silica gel 60, deprotected with trifluoroacetic acid/dimethyl sulfide/water [92/5/3 (v/v/v), 3 h at 25 °C] and finally purified by reverse-phase high-pressure liquid chromatography as described above. The unalkylated peptide Bimta-GK_5SKTKC-OMe was prepared in an analogous manner, using an *S*-trityl group to protect the cysteine thiol residue until the final deprotection step.

Methods. Lipid mixtures for vesicle preparation were dried down in chloroform/methanol 2:1 [including 1% (v/v) water for mixtures containing PIP or PIP_2], dried under nitrogen and then under high vacuum (for >4 h) and vortexed into buffer (140 mM KCl, 10 mM NaCl, 5 mM MOPS, and 0.5 mM EDTA, pH 7.2, except where otherwise indicated). The resuspended lipids were extruded through 0.1-μm polycarbonate filters after repeated freeze/thawing (*21*).

Peptide Transfer-Kinetics Assay. "Donor" lipid vesicles incorporating the fluorescence quencher DABS-PC (2 mol %) were prepared as outlined above and incubated with fluorescent peptide (at a 1:800 peptide/lipid molar ratio) for 15 min at 37 °C. "Acceptor" lipid vesicles were prepared with the same lipid composition but omitting DABS-PC. To examine the kinetics of intervesicle peptide transfer, donor vesicles (normally 30 nmol of lipid) in 3 mL of buffer were first dispensed into the fluorometer cuvette at 37 °C. The time course of dequenching of peptide fluorescence upon subsequent addition of acceptor vesicles (normally 300 nmol of lipid) was monitored and analyzed as described previously to determine the kinetics of intervesicle peptide transfer (*18*).

Peptide Lipid-Partitioning Assay. Lipid vesicles prepared as above and containing 2 mol % DABS-PC were incubated with fluorescent peptides (1:800 or 1:400 mol/mol of lipid) for 15 min at 37 °C. Aliquots of this mixture (10−30 nmol of lipid, the amount remaining constant within any one experiment), were injected at 37 °C into 3 mL of buffer in a stirred fluorimeter cuvette (λ_{ex} = 390 nm, λ_{em} = 468 nm) containing varying amounts of the same lipid vesicles without peptide (0−2000 nmol). After subtraction of the vesicle light-scattering signal and correction for inner-filter effects due to DABS-PC absorbance (see below), the final peptide fluorescence (F) was fitted to

$$F = F_0 - \Delta F_{max}\left(\frac{[L_{exp}]}{K_d^{eff} + [L_{exp}]}\right) \quad (1)$$

where $[L_{exp}]$ is the concentration of *surface-exposed* vesicle lipids, K_d^{eff} is the dissociation constant for the peptide−vesicle interaction (*20*), and F_0 and ($F_0 - \Delta F_{max}$) represent the corrected fluorescence values for the same quantity of

[1] Abbreviations: DABS-PC, 1-palmitoyl-2-[12-[[[4-[(4-(dimethylamino)phenyl)azo]phenyl]sulfonyl]methylamino]stearoyl]phosphatidylcholine; EDTA, ethylenediaminetetraacetic acid, trisodium salt; MOPS, 3-(*N*-morpholino)propanesulfonic acid, sodium salt; MARCKS, myristoylated alanine-rich C-kinase substrate protein; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)phosphatidylethanolamine; PC, phosphatidylcholine (from egg yolk except where otherwise indicated); PE, phosphatidylethanolamine prepared by transphosphatidylation from egg yolk phosphatidylcholine; PI, bovine liver phosphatidylinositol; PIP, phosphatidylinositol-4′-phosphate; PIP_2, phosphatidylinositol-4′, 5′-bisphosphate; PS, 1,2-dioleoylphosphatidylserine; TLC, thin-layer chromatography. Peptides are designated using the one-letter amino acid code and the following additional abbreviations: Boc, *N-tert*-butyloxycarbonyl; Bimta-, S-bimanylmercaptoacetyl-; Fmoc, *N*-fluorenylmethoxycarbonyl; StBu, *tert*-butylmercapto.

peptide at zero and at saturating lipid concentrations, respectively. Fluorescence values were corrected for inner-filter effects (due primarily to DABS-PC absorbance) by parallel measurements using the same lipid vesicles together with the peptide Bimta-GK_3S-OH, which did not significantly bind to the vesicles at the lipid concentrations used here.

For technical reasons it was not feasible to determine directly for each batch of lipid vesicles the quantity $[L_{exp}]$ in eq 1. Instead, parallel dispersions of vesicles were prepared omitting DABS-PC, and the fraction of vesicle aminophospholipids exposed at the vesicles' outer surface (which for large vesicles equals the fraction of surface-exposed aminophospholipids) was determined by the trinitrobenzenesulfonic acid assay (*18*, *22*). For vesicles of a variety of compositions the fraction of surface-exposed aminophospholipids consistently fell in the range 0.43−0.48 (mean 0.46). For vesicles containing zero or very high levels of aminophospholipids, 0.5 mol % NBD-PE was included in the vesicle lipids and the fraction of surface-exposed lipids was estimated as the fraction of fluorescent lipid rapidly reduced by 5−10 mM dithionite (*23*), yielding estimates very similar to those noted above. We therefore estimated the fraction of surface-exposed lipids for all vesicle preparations as 0.46 times the total lipid concentration, determining the latter by the procedure of Lowry and Tinsley (*24*).

Binding of the unalkylated peptide Bimta-GK_5SKTKC-OMe to lipid vesicles was of too low affinity to monitor using the above assay and was measured instead by the centrifugation-based assay of Buser et al. (*25*), again using 140 mM KCl, 10 mM NaCl, 5 mM MOPS, and 0.5 mM EDTA, pH 7.2, as the incubation buffer but adding 1 mM dithiothreitol to prevent cysteine oxidation.

Vesicle-Competition Assays. Donor lipid vesicles incorporating DABS-PC (2 mol %) and preincubated with fluorescent peptide (1:800 mol of peptide/mol of lipid) as well as acceptor vesicles (lacking DABS-PC and peptide and normally with a different lipid composition) were prepared as described above for the peptide transfer-kinetics assay. To assay the relative affinity of the fluorescent peptide for the donor vs the acceptor vesicles, 30-nmol portions of peptide-loaded donor vesicles were added to variable amounts of acceptor vesicles in 3 mL of buffer while the fluorescence was continuously monitored. The resulting final fluorescence values, corrected for vesicle light-scattering and small inner filter effects (the latter determined using the same vesicles with the nonadsorbing peptide Bimta-GK_3S-OH), were fitted to

$$F = F_0 + \Delta F_{max}\left(\frac{[L_{exp}]_A}{[L_{exp}]_A + K_{rel}(D/A)[L_{exp}]_D}\right) \quad (2)$$

where $[L_{exp}]_A$ and $[L_{exp}]_D$ are respectively the concentrations of surface-exposed lipids presented by the acceptor and donor vesicles, $K_{rel}(D/A)$ is the relative affinity of the peptide for the donor over the acceptor vesicles, and F_0 and ΔF_{max} represent respectively the peptide fluorescence in the presence of donor vesicles only or of a large excess of acceptor vesicles.

RESULTS

In Figure 1 are shown the structures of the fluorescent lipidated peptides examined in this study, based on the carboxy-terminal sequence of K-*ras*4B from *Xenopus laevis* (*26*).[2] As our efforts to prepare the farnesylated full-length peptide did not yield a product of satisfactory purity, we instead prepared the peptide modified with an *S*-undecyl group of equivalent hydrophobicity (ref *20* and this report), designated Bimta-GK_5SKTKC(undecyl)-OMe. By use of this peptide and shorter *S*-farnesyl and *S*-undecyl derivatives, the affinity of binding of the K-*ras*4B carboxy-terminal sequence to lipid vesicles of varying compositions could be accurately assessed. A bimanyl group was used as the fluorescent label because it does not contribute detectably to the interactions of labeled peptides with lipid bilayers (*20*, *27*).

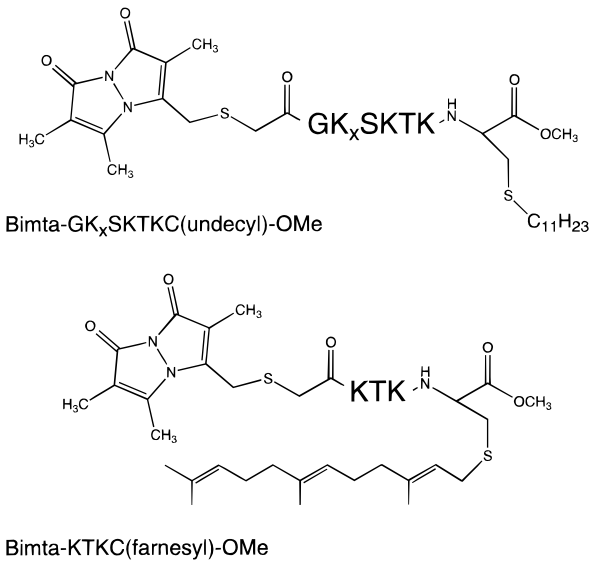

FIGURE 1: Structures of the fluorescent lipidated peptides examined in this study.

Kinetics of Peptide Desorption from Lipid Vesicles. In preliminary experiments we examined the rate of desorption of peptides based on the K-*ras*4B carboxy-terminus from lipid bilayers (large unilamellar lipid vesicles) of varying composition and surface charge as described in Materials and Methods. The peptide was first bound to donor vesicles containing the nonexchangeable fluorescence quencher DABS-PC (*18*), and upon mixing with a 10-fold excess of quencher-free acceptor vesicles the rate of peptide transfer from the donor to the acceptor vesicles was determined from the time course of fluorescence enhancement.

As shown in Figure 2, as the molar percentage of PS in PC vesicles increases, the rate of intervesicle transfer of Bimta-GK_5SKTKC(undecyl)-OMe gradually decreases. However, even for vesicles containing 40 mol % PS the half-time for intervesicle transfer of the labeled peptide is less than 1 min.. Relatively rapid kinetics were also observed for transfer of the peptide between vesicles containing up to 25 mol % PS in 1:1 (molar proportions) PE/PC (not shown). The shorter peptides Bimta-KTKC(undecyl)-OMe and Bimta-KTKC(farnesyl)-OMe likewise showed very rapid intervesicle transfer in these systems (half-times less than the

[2] The carboxy-terminal sequences of human and murine K-*ras*4B, otherwise identical to that of the *X. laevis* protein, contain six rather than five contiguous lysine residues (*60*−*62*). As discussed in the text, this difference appears to have relatively little effect on either the biophysical or the targeting properties of this sequence.

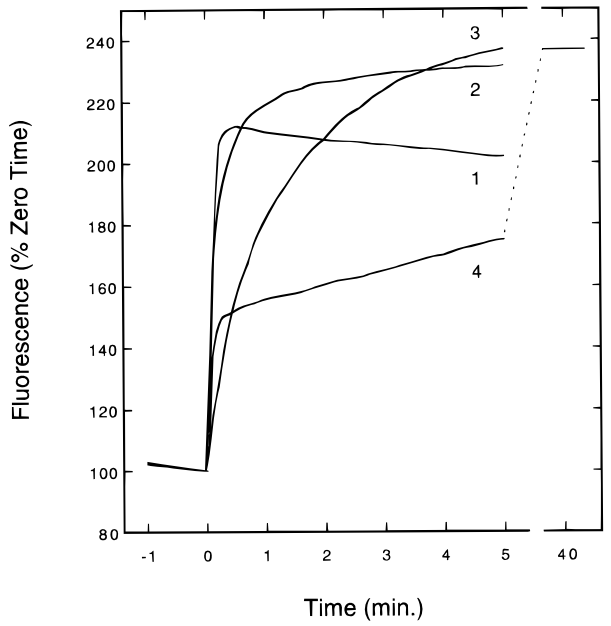

FIGURE 2: Time courses of intervesicle diffusion of the peptide Bimta-GK_5SKTKC(undecyl)-OMe at 37 °C between donor (quencher-incorporating) and acceptor (quencher-free) large unilamellar lipid vesicles composed of 20:80 PS/PC (curve 1), 30:70 PS/PC (curve 2), 40:60 PS/PC (curve 3) or 20:50:30 PS/PE/PC (curve 4). Other experimental details were as described in the text.

mixing time of ca. 2−3 s.). The halftime for intervesicle transfer of the peptides was not significantly affected by varying the concentration of acceptor vesicles, consistent with previous findings that at these low lipid concentrations intervesicle transfer of similar amphiphiles does not proceed via intervesicle collisions (*18*, *28*, *29*). Control experiments using donor and acceptor vesicles labeled with the fluorescent phospholipids NBD-PE and rhodaminyl-PE, respectively (*30*), revealed no intervesicle transfer of lipids on this time scale in the presence or absence of the labeled peptide (not shown).

As shown in Figure 2 (curve 4), vesicles containing high proportions of total aminophospholipids (e.g., 20:50:30 PS/PE/PC) showed comparatively slow transfer of a fraction of the vesicle-associated peptide molecules. However, in the presence of the peptide such vesicles also showed substantial aggregation, as indicated by a strong increase in turbidity that was not observed for vesicles with lower aminophospholipid contents. The slowly transferring population of peptides just noted thus likely represents peptide molecules trapped within aggregates of donor vesicles. Taken all together, the above results indicate that spontaneous dissociation of the K-*ras*4B carboxy-terminal sequence from lipid bilayers of physiological surface charge is a rapid process (half-times of tens of seconds or faster).

Previous work has indicated that in mammalian cells the cytoplasmic face of the plasma membrane contains PE and PC in a molar ratio near or slightly greater than unity (*31*) and that the plasma membrane contains 15−20 mol % total anionic lipids, the majority of which appear to be present on the cytoplasmic side of this membrane (*31*, *32*). Accordingly, most of the peptide/vesicle partitioning and vesicle-competition experiments described in the sections below were carried out using vesicles combining various mole fractions of anionic lipids with equimolar PE and PC. In light of the results discussed above, vesicles combining PE and PC in molar ratios greater than 1:1, or containing greater than 25 mol % anionic lipids in combination with PE/PC, were avoided in order to avoid potential artifacts associated with peptide-induced vesicle aggregation.

Vesicle/Aqueous Partitioning of K-ras4B Carboxy-Terminal Peptides. The association of bimane-labeled peptides based on the K-*ras*4B carboxy-terminus with lipid vesicles containing low amounts of anionic lipids (≤10 mol %) could be readily monitored by the peptide lipid-partitioning assay described in Materials and Methods, using vesicles incorporating the energy-transfer quencher DABS-PC. In this assay a small amount of peptide (preincubated with a small amount of vesicles as carrier) was mixed with varying amounts of additional lipid vesicles of the same composition, and the final peptide fluorescence was determined as a function of the total lipid concentration. The resulting hyperbolic relationship, an example of which is shown in Figure 3A, was fitted to eq 1 in Materials and Methods to yield the effective dissociation constant K_d^{eff} for peptide−vesicle association, representing the concentration of *surface-exposed* vesicle lipids (to which the peptide has ready access) at which 50% of the peptide is vesicle-associated. As discussed previously, this parameter is related to the mole fraction-based partition coefficient for peptide−vesicle association, K_p, by the relationship $K_d^{eff} = (55.5\ M)/K_p$ (see refs *20* and *33*)[3].

As shown in Figure 4 (●), for association of Bimta-GK_5SKTKC(undecyl)-OMe with vesicles containing varying proportions of PS in 1:1 PE/PC the value of K_d^{eff} falls exponentially with increasing anionic lipid content, decreasing almost 50-fold from 0 to 10 mol % PS. The value of K_d^{eff} measured for peptide binding to 10:90 PS/PC vesicles (Figure 4, ▲) was very similar to that measured using 10:45:45 PS/PE/PC vesicles.

To assess the effect of substituting an *S*-undecyl for an *S*-farnesyl group on the bilayer-partitioning affinity of peptides derived from the K-*ras*4B carboxy-terminus, using the peptide lipid-partitioning assay we also compared the binding of the peptides Bimta-KTKC(farnesyl)-OMe and -KTKC(undecyl)-OMe to 5:47.5:47.5 PS/PE/PC vesicles. The values of K_d^{eff} thereby determined were 21.6 ± 4.7 μM for the farnesyl and 17.6 ± 2.3 μM for the undecyl peptide (mean of three experiments). The close correspondence of these values is consistent with previous findings for other *S*-farnesyl and *S*-undecyl peptides (*20*). The values of K_d^{eff} predicted for binding of the peptide Bimta-GK_5SKTKC-(farnesyl)-OMe to PS/PE/PC vesicles are thus very similar to (specifically, about 1.2-fold greater than) those shown for the *S*-undecyl peptide in Figure 4.

Vesicle Surface Charge Discrimination by the K-ras C-Terminal Peptide. At anionic lipid contents greater than about 10 mol %, Bimta-GK_5SKTKC(undecyl)-OMe bound to lipid vesicles too strongly to allow accurate determination of K_d^{eff} using the above assay. This limitation was circum-

[3] It should be noted that the effective dissociation constant K_d^{eff} as defined here does not correspond to a true dissociation constant as would be used to characterize the binding of a peptide to lipids with a fixed binding stoichiometry. However, since the partitioning isotherms measured here have the same hyperbolic form as a classical binding curve, the parameter K_d^{eff} provides a convenient means to characterize the affinity of association of peptide molecules with the vesicle surface.

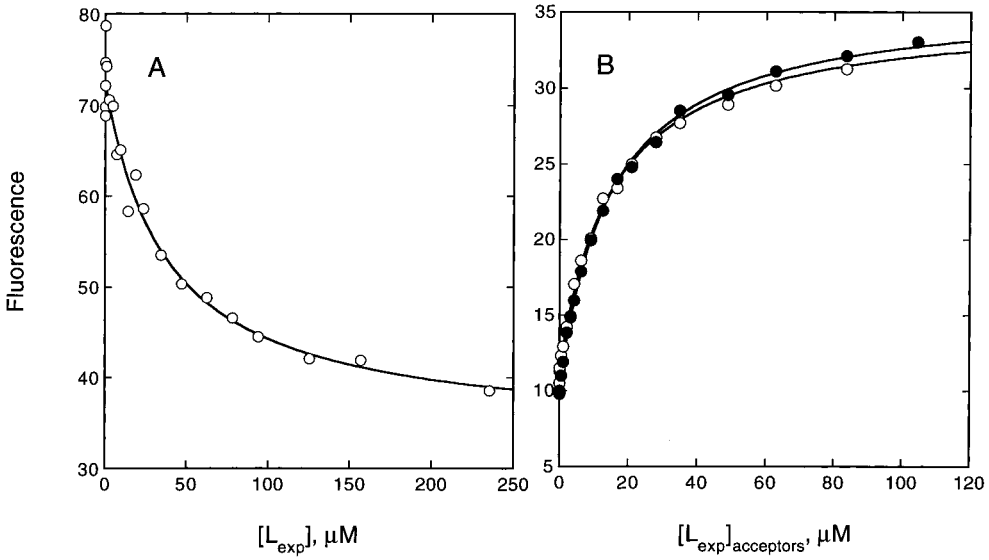

FIGURE 3: (A) Representative "binding curve", obtained using the peptide lipid-partitioning assay, for the association of Bimta-GK_5-SKTKC(undecyl)-OMe with 5:47.5:45.5:2 (molar proportions) PS/PE/PC/DABS-PC large unilamellar vesicles. Fitting the curve shown to eq 1 in the text gave a value of $K_d^{eff} = 37.9 \pm 7.3$ μM. (B) Representative "competition curve" measuring the distribution of Bimta-GK_5-SKTKC(undecyl)-OMe between donor and acceptor vesicles containing 13.5 and 10 mol % PS, respectively, in equimolar PE/PC; the PC fraction in the donor vesicles included 2 mol % DABS-PC. The data shown were obtained using either the standard buffer (140 mM KCl, 10 mM NaCl, 5 mM MOPS, and 0.5 mM EDTA, pH 7.2; ○) or the same buffer with 0.5 mM $MgCl_2$ in place of EDTA (●). From these data, fitted to eq 2 as shown, and the value $[L_{exp}]_{donors} = 4.21$ μM determined as described in the text, a value of $K_{rel} = 3.40 \pm 0.23$ (no Mg^{2+}) or 3.51 ± 0.13 (+ Mg^{2+}) was calculated for peptide binding to the donor over the acceptor vesicles in the experiment illustrated.

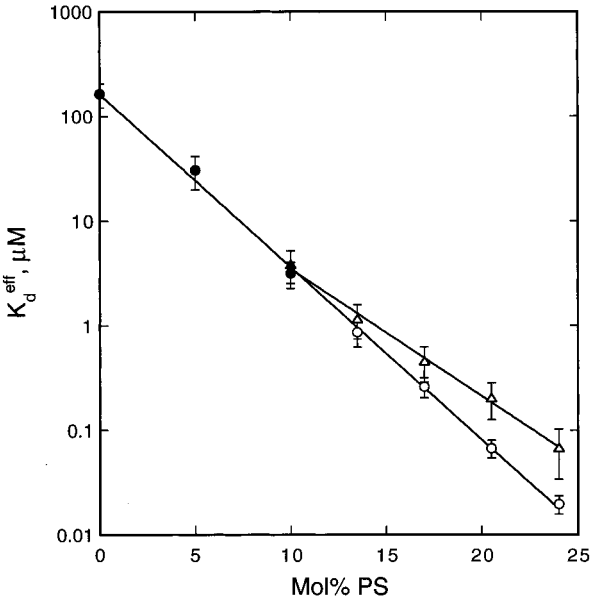

FIGURE 4: Variation of K_d^{eff} for binding of Bimta-GK_5SKTKC-(undecyl)-OMe to vesicles composed of the indicated mole fractions of PS in equimolar PE/PC (●, ○) or in PC (▲, △). Filled symbols represent values determined directly from the lipid-partitioning assay, while open symbols represent values determined by combining the results of lipid-partitioning and vesicle-competition assays as described in the text. Error estimates for K_d^{eff} values determined by the latter method were calculated by an appropriate propagation-of-error treatment. As discussed in the text, the predicted values of K_d^{eff} for the farnesylated peptide will be 1.2-fold higher than those shown for the *S*-undecyl peptide.

vented by using the vesicle-competition assay described in Materials and Methods. As illustrated in Figure 3B, when a fixed quantity of the peptide, initially bound to a constant amount of donor vesicles containing DABS-PC, is mixed with increasing amounts of quencher-free acceptor vesicles, the final fluorescence level increases hyperbolically.[4] From data like those presented in Figure 3B it is possible to determine the dimensionless parameter K_{rel}(D/A) representing the relative affinity of the peptide for the donor over the acceptor vesicles (see Materials and Methods). This parameter in turn simply equals the ratio of the dissociation constants K_d^{eff} for peptide binding to the acceptor vs the donor vesicles. Using vesicles of any two compositions A and B, by determining the value of K_d^{eff} for peptide binding to vesicles of composition A and the value of K_{rel}(A/B), we can thus calculate the absolute value of K_d^{eff} for peptide binding to vesicles of composition B from

$$K_d^{eff}(B) = K_d^{eff}(A)K_{rel}(A/B) \quad (3)$$

In control experiments (not shown) using donor and acceptor vesicles both composed of 10:45:45 (molar proportions) PS/PE/PC, the measured value of K_{rel} was unity within experimental error, as expected (range 0.85−1.13 in three experiments).

The approach just outlined was used to determine the value of K_d^{eff} for binding of Bimta-GK_5SKTKC(undecyl)-OMe to vesicles containing increasing proportions of PS in a background of 1:1 PE/PC. In a first set of experiments the value of K_d^{eff} measured for vesicles containing 10 mol % PS (determined by the peptide lipid-partitioning assay) and the value of K_{rel} determined (by the vesicle-competition assay) for vesicles containing 10 vs 13.5 mol % PS were used in eq 3 to calculate the value of K_d^{eff} for vesicles containing 13.5 mol % PS. This estimate was then combined with the value of K_{rel} determined for vesicles containing 13.5 vs 17 mol % PS (again using the vesicle-competition assay)

[4] The vesicle-competition assay requires that essentially all of the peptide be bound to either the donor or the acceptor vesicles at all donor/acceptor ratios. This condition was fulfilled in all the experiments described.

to calculate in the same manner the value of K_d^{eff} for vesicles containing 17 mol % PS; estimates of K_d^{eff} for vesicles containing higher levels of PS were obtained by successive iterations of this approach. The results of these experiments are summarized in Figure 4 (○), which shows a strong and exponential variation of K_d^{eff} with vesicle PS content from 0 to 24 mol % PS. The exponential nature of this variation is consistent with that observed experimentally, and predicted theoretically, for association of other cationic proteins and peptides with bilayers containing roughly 0−35 mol % anionic lipids (*34*, *35*).

From the data shown in Figure 4 we can calculate that Bimta-GK_5SKTKC(undecyl)-OMe will discriminate by a factor of 6.7-fold in its binding to vesicles differing by 5 mol %, and by 45-fold between vesicles differing by 10 mol %, in PS content. The degree of discrimination between vesicles of different PS content was not significantly affected when 0.5 mM magnesium, a value comparable to the cytoplasmic free magnesium concentration in mammalian cells (*36*, *37*), was included in the assay buffer and EDTA was omitted (Figure 3B, filled symbols). The peptide Bimta-GK_3SKTKC(undecyl)-OMe showed only a modestly lower extent of discrimination between vesicles of different PS content than does Bimta-GK_5SKTKC(undecyl)-OMe; in a representative experiment using donor vesicles containing 13.5 mol % PS and acceptor vesicles containing 10% PS in equimolar PE/PC, this peptide gave a K_{rel} value of 2.63 ± 0.16 vs 3.06 ± 0.16 for Bimta-GK_5KTKC(undecyl)-OMe.

Also shown in Figure 4 are the results of a series of determinations of K_d^{eff}, estimated by the stepwise approach outlined above, for association of Bimta-GK_5SKTKC-(undecyl)-OMe with vesicles containing only PS and PC. It can be seen that the dissociation constant again varies exponentially with the mole percentage of PS, although for these vesicles the dependence is somewhat less steep; an increase of 5 mol % in the PS content of PS/PC vesicles decreases K_d^{eff} by only 4.1-fold vs 6.7-fold for PS/PE/PC vesicles.

Electrostatic vs Hydrophobic Contributions to Peptide−Vesicle Binding. The value of K_d^{eff} for association of Bimta-GK_5SKTKC(undecyl)-OMe with vesicles containing 24:38:38 (molar proportions) PS/PE/PC, estimated as described above, is 20 ± 4 nM, while the measured value of K_d^{eff} for binding of this peptide to 50:50 PE/PC vesicles is 163 ± 42 μM (Figure 4). Using these two values, we can calculate the electrostatic contribution to the binding of the lipidated peptide to 24:38:38 (molar proportions) PS/PE/PC vesicles to be $-RT \ln (163/0.02) = -5.6$ kcal mol^{-1}. By use of a centrifugation-based assay as described in Materials and Methods, the corresponding unalkylated peptide Bimta-GK_5-SKTKC−OMe was found to associate with vesicles of the same composition with a K_d^{eff} value of 4.7 ± 0.2 mM [mean of two independent experiments (not shown)], corresponding to a free energy of partitioning of $-RT (\ln K_p) = -RT \ln (55.5 \text{ M}/4.7 \text{ mM}) = -5.8$ kcal mol^{-1}. The close correspondence of these two free energies is expected when the interaction of the amino acid residues of the peptide with the membrane surface is largely electrostatic and when the hydrophobic and charged portions of the lipidated peptide are not widely separated (*25*). The hydrophobic contribution to binding of the farnesylated K-*ras*4B carboxy-terminal peptide to lipid bilayers can then be estimated as roughly $-RT \ln [4.7 \text{ mM}/[(20 \text{ nM})(1.2)]] = -7.5$ kcal mol^{-1}, using the values of K_d^{eff} measured for the *S*-undecyl and the unalkylated peptide and the 1.2-fold difference in lipid-binding affinity measured above for the farnesyl vs the *S*-undecyl group.

Effects of Cholesterol on Peptide−Vesicle Binding. The plasma and certain other membranes of mammalian cells contain high levels of cholesterol, while others contain much lower levels of sterol (*38*). Using the vesicle-competition assay, we therefore examined the relative affinity of Bimta-GK_5SKTKC(undecyl)-OMe for PE/PC/PS (10:45:45 or 20:40:40 molar proportions) vesicles containing either 0 or 33 mol % cholesterol. The values of K_{rel} estimated in such experiments, based on *total* vesicle lipid (i.e., phospholipid plus sterol) concentrations, were 1.36 ± 0.13 for 10:45:45 PS/PE/PC vesicles in competition with 10:45:45:50 PS/PE/PC/cholesterol vesicles and 3.26 ± 1.13 for 20:40:40 PS/PE/PC vesicles in competition with 20:40:40:50 PS/PE/PC/cholesterol vesicles.

The effects of cholesterol incorporation on binding of Bimta-GK_5SKTKC(undecyl)-OMe to PS/PE/PC vesicles can be satisfactorily explained on the basis of reduction of the vesicle surface charge upon sterol incorporation, as the following calculation will demonstrate. Using data reported for POPC/cholesterol monolayers at surface pressures comparable to those expected for bilayers (*39*) and assuming generally similar behavior for the lipid mixtures examined here, we can estimate that the addition of 33 mol % cholesterol to a given PE/PC/PS mixture will increase the total surface area by roughly 14% and decrease the surface charge density by roughly 12%.[5] The surface charge of 10:45:45:50 (molar proportions) PS/PE/PC/cholesterol vesicles is thus estimated to be equivalent to that of PS/PE/PC vesicles containing ca. 8.8 mol % PS, which from Figure 4 are predicted to bind Bimta-GK_5SKTKC(undecyl)-OMe roughly 1.6-fold more weakly than PS/PE/PC vesicles containing 10 mol % PS. Based on surface-charge effects alone we therefore predict that 10:45:45:50 PS/PE/PC/cholesterol vesicles will likewise bind the peptide with ca. 1.6-fold lower affinity than 10:45:45 PS/PE/PC vesicles, and (using similar reasoning) that 20:40:40:50 PS/PE/PC/cholesterol vesicles will bind the peptide with ca. 2.6-fold lower affinity than 20:40:40 PS/PE/PC vesicles. These predicted values are in satisfactory agreement with the experimental estimates of K_{rel} noted above (1.36 ± 0.13 and 3.26 ± 1.13, respectively, in favor of the cholesterol-free vesicles), suggesting that cholesterol modulates the affinity of peptide−vesicle partitioning primarily by diluting the vesicle surface charge.

Anionic Lipid Selectivity of the K-ras C-Terminal Peptide. The vesicle-competition assay was also employed to assess the relative affinity of binding of Bimta-GK_5SKTKC-(undecyl)-OMe to vesicles containing other anionic lipids in addition to PS. These experiments used donor vesicles containing 10 mol % PS plus 1.5−3.5 mol % of a second

[5] The predicted area increase due to the incorporation of 33 mol % cholesterol was calculated from the monolayer data of Smaby et al. (*39*), who reported a molecular area of 36.8 Å^2 for cholesterol and a reduction of approximately 15% in the molecular area for the phospholipid component at a surface pressure of 30−35 mN/m, assuming a molecular area of 64 Å^2 for the phospholipids in the absence of cholesterol.

Table 1: Discrimination by Bimta-GK_5SKTKC(undecyl)-OMe between Acceptor Vesicles Containing 10 mol % PS and Donor Vesicles Containing 10 mol % PS plus a Second Anionic Lipid[a]

second anionic lipid	estimated net charge	K_{rel}(exptl)[b]	K_{rel}(predicted)[c]
PI (3.5 mol %)	−1	3.40 ± 0.13	3.8
PI-4′-phosphate (1.5 mol %)	−2 to −3	2.60 ± 0.39	3.1−5.5
PI-4′,5′-bisphosphate (2 mol %)	−3 to −3.5	14.71 ± 3.53	9.8−14.4
phosphatidic acid (2 mol %)	−1. 4 to −1.5	3.84 ± 0.06	2.9−3.1

[a] Donor vesicles were prepared containing 2 mol % DABS-PC and the indicated proportions of anionic lipids in equimolar PE/PC; acceptor vesicles were prepared without DABS-PC and containing 10 mol % PS only in equimolar PE/PC. [b] Tabulated values represent the mean (± SD) of triplicate experiments, analyzed as described in the text. [c] Values of K_{rel} predicted from surface-charge effects as described in the text.

anionic lipid and acceptor vesicles containing 10 mol % PS alone, both in an equimolar PE/PC background; the relative peptide-binding affinity (K_{rel}) for these donor/acceptor pairs was determined as described above.

As shown in Table 1, vesicles containing a second anionic lipid in addition to 10 mol % PS in all cases bound Bimta-GK_5SKTKC(undecyl)-OMe with higher affinity than did vesicles containing 10 mol % PS only. To evaluate the contribution of surface-charge effects to these results, using the expected net charges of each of the anionic phospholipids at physiological pH (*40*−*43*) and the results shown in Figure 4, for each donor−acceptor vesicle pair listed in Table 1 we calculated the value of K_{rel} expected if the second anionic lipid enhances peptide binding only through its surface-charge contribution. Comparison of the predicted values of K_{rel} (Table 1, fourth column) with the experimental values (third column) shows close agreement in most cases, and in no case does the presence of the second anionic lipid enhance peptide binding by a factor substantially greater than that predicted on the basis of surface-charge effects alone. It thus appears that the polybasic carboxy-terminal sequence of K-*ras*4B discriminates between bilayers with different anionic lipid compositions purely on the basis of their surface charge and not by associating specifically with particular anionic lipids.

Since PI is a relatively abundant anionic lipid in membranes, we also tested whether Bimta-GK_5SKTKC(undecyl)-OMe discriminates between vesicles containing 10 mol % PI vs 10 mol % PS in equimolar PE/PC. The value of K_{rel}(PI/PS) = 1.18 ± 0.16 thereby determined again indicates no significant discrimination between these two anionic phospholipids, as concluded above.

DISCUSSION

The present results indicate that the carboxy-terminal polybasic region of K-*ras*4B associates with lipid bilayers of physiological anionic lipid content with high affinity but in a rapidly reversible manner, with a half-time of seconds or less for the dissociation from the lipid surface. This conclusion agrees with the finding of Yokoe and Mayer (*44*) that a green fluorescent protein/K-*ras*4B fusion protein shows a strong but rapidly reversible interaction with the plasma membrane in mammalian cells. The present results suggest that no special protein factor is needed to promote this rapid reversibility of K-*ras*4B association with the plasma membrane lipid bilayer. Even a geranylgeranylated form of K-*ras*4B, as appears in cells treated with farnesyltransferase inhibitors (*45*−*49*), would be predicted to exhibit a readily reversible association with lipid bilayers of physiological anionic lipid content (half-times of at most several minutes for bilayer dissociation) based on the present results and our previous studies of the kinetics of interbilayer transfer of farnesylated vs geranylgeranylated peptides (*18*).[6]

The polybasic carboxy-terminal sequence of K-*ras*4B discriminates strongly between lipid membranes of different surface charge, as has been observed for the polybasic regions of other lipidated proteins such as $p60^{src}$ and MARCKS (*25*, *50*−*52*). The degree of surface charge discrimination observed for the K-*ras*4B carboxyl-terminal peptide (net charge +7) using PC/PS vesicles is intermediate between that observed previously using PC/PS or PC/phosphatidylglycerol vesicles with either pentalysine or the myristoylated N-terminal peptide of $p60^{src}$ (net charge +5) and that reported for a peptide of net charge +13 corresponding to the polybasic region of MARCKS (*25*, *34*, *35*, *51*, *52*).[7] Our present findings indicate that the carboxy-terminal sequence of K-*ras*4B exhibits a significantly greater degree of surface charge-based discrimination between different bilayers when the bilayers contain physiological proportions of PE. The basis of this effect is presently unclear, although Kim et al. (*51*) have likewise noted that the neutral lipid composition of mixed anionic/neutral lipid vesicles can significantly affect the degree to which binding of the MARCKS protein varies with the vesicle surface charge. Under physiological ionic conditions, when presented with two PS/PE/PC bilayers that differ by 10 mol % in PS content (or by an equivalent amount in surface charge), the peptide Bimta-GK_5SKTKC(undecyl)-OMe would show a 45-fold greater accumulation in the more negatively charged bilayer; if the bilayers were composed of PS and PC alone the degree of discrimination would be roughly half as great.

Comparison of the surface charge-discriminating properties of the peptides Bimta-GK_5SKTKC(undecyl)-OMe (net charge +7) and Bimta-GK_3SKTKC(undecyl)-OMe (net charge +5) reveals only a modest reduction in the degree of surface-charge discrimination when two of the contiguous lysine residues are removed. Data reported by Ghomaschi et al. (*53*) for a K-*ras*4B-derived peptide containing six contiguous lysine residues (net charge +8) suggest that this peptide shows a degree of charge discrimination between PC/phosphatidylglycerol vesicles that is very similar to that observed here using Bimta-GK_5SKTKC(undecyl)-OMe with PC/PS vesicles.[8] These findings are consistent with previous reports that up to three of the lysine residues in the -K_6- sequence of murine K-*ras*4B can be mutated to glutamine

[6] Previous results indicate that the replacement of a farnesyl by a geranylgeranyl residue will decrease the rate of bilayer desorption of a prenylated peptide by roughly 25-fold (*18*). Combining this result with our present kinetic data, we predict that the geranylgeranylated carboxy-terminus of K-*ras*4B would desorb from lipid bilayers containing 30 mol % or less anionic phospholipid with a half-time on the order of 10 min or less under physiological conditions.

[7] Using data provided in the cited papers, we can roughly estimate that at ionic strength 0.15 M the MARCKS peptide would discriminate by a factor of at least 200-fold, and the *src* peptide and pentalysine by roughly 7−8-fold, between two PS/PC bilayers differing by 10 mol % in anionic lipid content.

residues without detectably impairing the plasma-membrane targeting function of this region (*2*, *4*).

While the polybasic carboxy-terminal sequence of K-*ras*4B discriminates strongly between bilayers of different surface charge, this region of the protein shows no evidence of specific binding to any of the common mono- and polyanionic phospholipids found in the cytoplasmic face of the plasma membrane. While we have not examined the binding of the K-*ras*4B carboxy-terminal peptide to PI-3′,4′-bisphosphate and PI-3′,4′,5′-trisphosphate, these latter lipids appear unlikely to play a primary role in targeting K-*ras*4B to the plasma membrane, as they are present at negligible levels in unstimulated cells (*54*), while the plasma membrane association of K-*ras*4B does not seem to be dependent on cellular activation.

Implications for K-ras4B Targeting to the Plasma Membrane. The farnesylated/polybasic carboxyl-terminal sequence of mature K-*ras*4B has been shown to function as an autonomous localization domain that can target a variety of heterologous proteins to the plasma membrane (*3*, *9*−*12*). As discussed in the introduction, it remains unresolved whether the plasma membrane-targeting function of the mature K-*ras*4B carboxy-terminal sequence rests on interactions with a putative plasma membrane receptor specific for this sequence or with plasma membrane lipids. Our results suggest that if the lipid hypothesis is correct, the targeting function of this sequence must rest on thermodynamic rather than kinetic factors and on a surface potential-sensing function of this sequence rather than on specific interactions with particular anionic phospholipids in the plasma membrane.

Assessing the plausibility of a surface charge-sensing mechanism for K-*ras*4B localization to the plasma membrane will require knowledge of two parameters that are presently unknown: first, by exactly what factor K-*ras*4B is enriched in the plasma membrane relative to other cellular membranes, and second, the degree to which the surface potential of the cytoplasmic aspect of the plasma membrane (or of particular regions therein) may differ from that of the cytoplasmic faces of other cellular membranes. Our present data suggest that K-*ras*4B could be concentrated to a 50-fold (100-fold) greater surface density at the plasma membrane than on other cellular membranes if the plasma membrane contains 10 mol % (12 mol %) more monoanionic lipids at its cytoplasmic face (or local regions within it), or exhibits an equivalent difference in surface charge, compared to intracellular membranes. This possibility is unfortunately difficult to evaluate using presently available data. The plasma membrane of mammalian cells does not appear to contain a dramatically higher level of anionic lipids than do other cellular membranes (*32*). However, anionic phospholipids as a class appear to be concentrated at the cytoplasmic face of the plasma membrane (*55*), and recent studies have raised the possibility that at least some anionic lipid species may be enriched in lateral domains within the cytoplasmic face of this membrane (*56*−*59*). Less is known about the transverse (or lateral) distributions of anionic lipids within intracellular membranes, although at least some intracellular membranes, such as that of the endoplasmic reticulum, may exhibit a more symmetrical transbilayer distribution of anionic phospholipids than does the plasma membrane (*55*). Better characterization of the lipid compositions and lateral distributions, and/or direct comparisons of the electrostatic potential, at the cytoplasmic surfaces of the plasma and other cellular membranes under physiological conditions will help to assess the plausibility of a surface-charge-based mechanism for the plasma membrane targeting of the K-*ras*4B carboxy terminus.

[8] The data of Ghomaschi et al. (*53*) suggest that at an ionic strength of 0.1 M the peptide AcC(maleimido)GK_6SKTKC(farnesyl)-OMe will show an 18-fold enhancement in binding affinity for PC/PG vesicles when the anionic lipid content is increased by 10 mol %, assuming an exponential variation of the binding affinity with the vesicle anionic lipid content. For the peptide Bimta-GK_5SKTKC(undecyl)-OMe, at ionic strength 0.15 M and using PC/PS vesicles (this study), the corresponding figure is 17-fold.

ACKNOWLEDGMENT

We thank Drs. Stuart McLaughlin and David Daleke for useful discussions.

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BI973077H