ACKNOWLEDGMENTS

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Registry No. ATP, 56-65-5; DNA topoisomerase, 80449-01-0.

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Normal Cellular Ha *ras* p21 Protein Causes Local Disruption of Bilayer Phospholipid

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ABSTRACT: We have investigated the interactions of the p21 protein of c-Ha *ras* with its phospholipid environment. Gel filtration of detergent-"solubilized" p21 revealed that this preparation consisted of a mixture of multimolecular aggregates of protein and phospholipid and also a population of individual p21 molecules. Addition of 8 M urea to p21 preparations increased the solubility of the molecule in detergent solutions upon the removal of this denaturant. The progressive addition of the detergent cholate appeared to increase the efficiency of p21 preparations to bind GTP. This affinity for GTP was not removed even at high detergent concentrations, when delipification of the p21 was presumably effected. Modification of the composition of the phospholipid species surrounding the protein did not appear to alter its affinity for GTP. Electron spin resonance studies with membrane spin-labels indicated a perturbation of the bilayer extending to between 44 and 100 phospholipids surrounding the molecule. However, no evidence was found for any population of intimately bound phospholipid, which is seen as an annulus of about 30 lipids in transmembrane proteins such as Ca^{2+}-ATPase. From these results we propose that the Ha *ras* p21 protein has the ability to associate directly with the membrane in a manner clearly discernible from that of a transmembrane protein.

Many membrane-bound proteins rely on interactions with their phospholipid environment to maintain correct functional activity (Montgomery et al., 1985; Hesketh et al., 1976; Duran & Cabib, 1980). The *ras* gene product p21 is known to reside in the cytosolic face of the plasma mebmrane and to have an intrinsic GTPase activity (Willingham et al., 1980; Lacal et al., 1986). Numerous recent studies have shown that p21 mediates the transduction of signals from receptor molecules within the membrane, to regulate enzymes controlling the production of second messenger molecules, such as phospho-

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diesterase. The *ras* protein therefore acts as a G-protein to pass extracellular signals to proteins which regulate cellular metabolism (Wakelam et al., 1986).

Mutations in *ras* genes are found in several types of tumor and presumably lead to the corruption of the regulatory control by p21 (Brown et al., 1986; Yuasa et al., 1983; Zarbl et al., 1985; Bos et al., 1985). If the biochemical significance of *ras* is to be clearly understood, it is vital that the precise location of the active p21 protein in the cell be known. Moreover, not only do many of the immunological tests for *ras* protein localization rely on the presence of p21 outside the cell membrane, but paradoxes concerning the solubility of p21 itself are apparent in the literature. The preferred environment of *ras* p21 is therefore an important question.

In this paper we have attempted to probe the interactions of p21 with its lipid environment both by modification of the composition of its associated phospholipid and also by probing changes in the membrane fluidity of this lipid by ESR.[1] Each of these approaches indicates differences with the behavior of p21 in comparison of that of similarly studied transmembrane proteins.

Materials and Methods

Growth of Cultures and Protein Extraction. Escherichia coli containing the c-Ha *ras* gene on the plasmid pJCL33 (Lacal et al., 1984) was used to express p21 by the thermoinducible synthesis of the protein initiated by heat shock at 42 °C as described by Lacal et al. (1984).

Cell pellets were resuspended in 5 times their volume of 30 mM Tris-HCl, pH 7.5, 0.1 M EDTA, 1 mM PMSF, 1% octyl glucoside, and 0.2% lysozyme and sonicated until a visible reduction in turbidity was noted. This crude p21 preparation was usually partially purified by repeatedly suspending the protein in Tris-HCl, pH 7.5, and 1% octyl glucoside and taking the pellet from these solutions obtained at 12000*g*.

Vesicle Preparations. Vesicles of specific phospholipid species for GTP binding experiments were prepared essentially as described previously (Montgomery & Gooday, 1985). The vesicles used in the ESR studies were sonicated with p21 preparations under nitrogen at 45 °C in 50 mM Tris-HCl buffer until a homogeneous suspension was formed.

The p21 used in the ESR experiments was prepared by two methods: (a) p21 from the cell lysate was sonicated successively with 0.5% octyl glucoside (3 times), 0.5% cholate (3 times), and 50 mM Tris-Hcl, pH 7.0 (3 times), the pellet from centrifugation at 12000*g* being retained throughout; (b) p21 was treated as in method a and in addition was delipified by extraction with chloroform–methanol (1:1 v/v), pelleted at 12000*g*, and resuspended in Tris-HCl, pH 7.0.

ESR Spectroscopy. ESR spectra were obtained with a Bruker ESP 300 spectrometer. Operating parameters were as follows: power 5 mW, modulation amplitude 1 G, and frequency 9.45 GHz. Temperature control was effected by control with a Bruker ER 4111 VT unit with the liquid nitrogen feed disconnected and replaced by nitrogen from a cylinder cooled through a heat exchanger in a dry ice/2-propanol bath. This provided better stability of control needed at the temperatures used for this biological material. The spin-label 5-doxylstearate was used to probe membrane fluidity, at a phospholipid:spin-label ratio of >100:1 to exclude the possibility of modification of the bilayer by the spin-label.

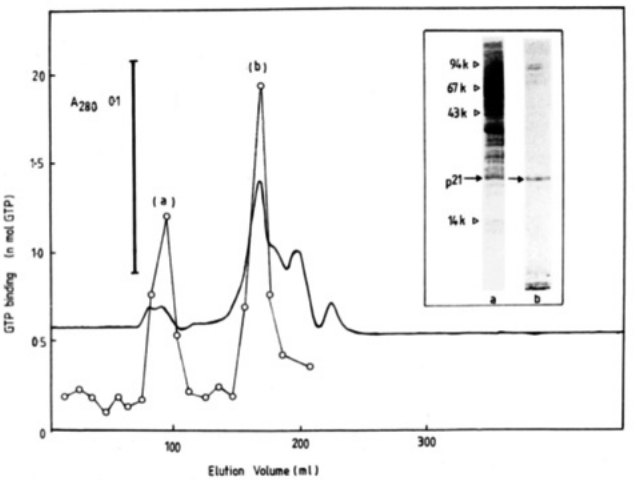

FIGURE 1: Elution profile of a 2-mL partially purified suspension of p21 (approximately 30 mg). GTP binding was assayed on 0.1-mL samples of fractions of column eluate. (Inset) SDS-polyacrylamide gel electrophoresis of lyophilized proteins of two GTP binding fractions.

Cholate Incubations. The cholate incubations were conducted as described previously (Montgomery & Gooday, 1985) for a similar form of "solubilized" protein suspension, and a 10-s sonication step was added prior to the GTP binding assay, due to the insoluble nature of this preparation. Binding was assayed in a volume of 0.5 mL, in reactions containing approximately 1 mg of p21 protein.

Phospholipid Displacement. The lipid coextracted with the p21 from the *E. coli* was progressively displaced by incubation with increasing quantities of specific lipid species, by a method described previously (Montgomery & Gooday, 1985). Incubations were 0.5 mL and contained about 1 mg of p21 protein. Results were expressed as percent of control values, typically representing about 40 pmol of GTP binding.

GTP Binding Assays. Samples (0.01–0.1 mL) were assayed from GTP binding by the addition of GTP binding buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.01% BSA, 2.5 mM ATP, and 0.01 mM GTP) containing 200 nCi of [^{3}H]GTP. The assay mixtures were incubated at 20 °C for 1 h and filtered through Millipore GSWP nitrocellulose filters. The filters were then washed with multiple 2-mL portions of nonradioactive "cold" GTP buffer to remove residual-free radioactive GTP; bound isotope was estimated by scintillation counting.

Protein Estimation and Electrophoresis. The Bradford (Bradford, 1976) and microbiuret (Gornall et al., 1949) assays were used to determine protein content, and average values from these two estimations are quoted throughout. SDS–polyacrylamide electrophoresis techniques were as described previously (Montgomery et al., 1984).

Gel Filtration. A column (80 × 1.6 cm) of the gel matrix Sephacryl S-200 Superfine was equilibrated and run in 50 mM Tris-HCl, pH 7.0, 5 mM dithiothreitol, 0.1% octyl glucoside, 0.5 M NaCl, and 0.02% sodium azide. The sample was 2 mL of p21 preparation sonicated against the above column buffer containing 1% octyl glucoside.

Results

Gel Filtration. The separation of the proteins of the crude p21 preparation by gel filtration indicated that the column eluate contained two fractions with high GTP binding: the

[1] Abbreviations: BSA, bovine serum albumin; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleylphosphatidylcholine; DPPE, dipalmitoylphosphatidylethanolamine; DPPS, dipalmitoylphosphatidylserine; 5-doxylstearate, stearate fatty acid moiety containing a 4,4-dimethyloxazolidine-*N*-oxyl (doxyl) ring; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; octyl glucoside, *n*-octyl β-D-glucopyranoside; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

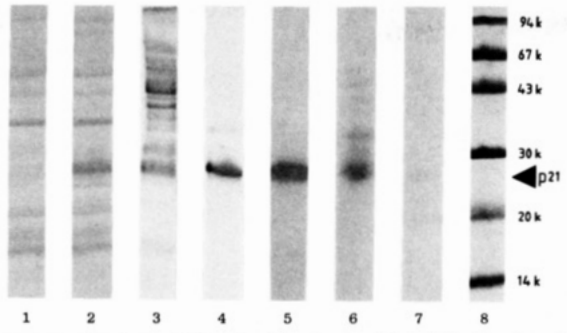

FIGURE 2: SDS–PAGE of variously treated p21, developed by Coomassie staining. (Lanes 1 and 2) Crude cell lysate from cell cultures at 30 °C (*ras* expression "off") and at 42 °C (*ras* expression "on"), respectively; (lanes 3 and 4) supernatent and pellet, respectively, from sonication of pellet from lane 2 with 1% digitonin. Material from lane 2 was sonicated with 1% octyl glucoside, the pellet was taken up in 8 M urea, and this supernatent was dialyzed against 0.5% deoxycholate (lane 5), 0.02% deoxycholate (lane 6), or distilled water (lane 7). Sonication of the crude p21 cell lysate (lane 2) with 1% octyl glucoside or 0.5% deoxycholate effected the same degree of purification as with 1% digitonin (lane 4). All further studies utilized p21 that had been thus purified by sonication with octyl glucoside, unless otherwise stated.

first with M_r >600K and the second with molecular weight of approximately 25K (Figure 1). SDS–polyacrylamide gel electrophoresis of these fractions indicated that both contained p21 (Figure 1, inset); the p21 preparation was therefore composed both of multimolecular aggregates containing protein which comigrated through the gel matrix and also of individual single p21 molecules.

Solubility Experiments. Attempts to solubilize the p21 by sonication in a range of membrane detergents, followed by centrifugation, led to the isolation of pellets that contained >90% p21 (Figure 2), and with the loss of only small amounts of protein. This partially purified protein was used as the starting material in the studies that follow. After p21 was dissolved in 8 M urea, dialysis could be used to maintain the p21 in dilute solutions of detergents such as octyl glucoside (4.5 mg/mL in a 1% solution) or deoxycholate (>20 mg in a 0.5% solution). Progressively less p21 remained in solution as the detergent was removed (Figure 2). All the solutions of p21 retained GTP binding affinity.

Cholate Incubations. The changes observed in GTP binding effected by the addition of increasing concentrations of cholate are indicated in Figure 3. This figure shows the p21 is either held in a more favorable conformation to bind GTP or is more effectively held in solution by the addition of 75 μg of cholate. If closed vesicles exist in this preparation, cholate addition would make these "leaky", thus increasing the exposure of p21 to GTP. However, as the p21 had already been exposed to extraction with 1% octyl glucoside it would seem reasonable to assume that such sealed vesicular p21 would represent no more than a minor fraction of the p21 protein, and hence would only slightly aid the initial increase in nucleotide binding by cholate.

The delipification of the p21 aggregates by this detergent does differ from results obtained with similar transmembrane proteins, such as chitin synthase or Ca^{2+}-stimulated ATPase (Montgomery et al., 1985; Hesketh et al., 1976), where enzymic activity is irreversibly lost as a result of delipification which occurs at detergent concentrations well below the highest used in Figure 3.

Phospholipid Displacement. In this experiment the phospholipid coextracted with the p21 from the *E. coli* was progressively displaced by the addition of specific phospholipid species. The results in Figure 4 are expressed as a percent of

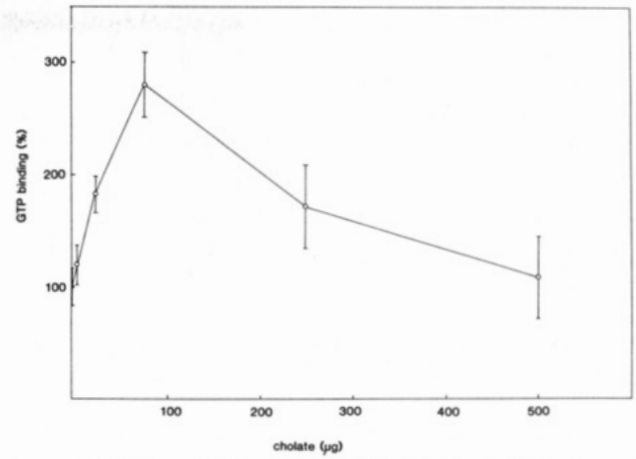

FIGURE 3: Effect of cholate on GTP binding of p21 (500 μL, containing about 1 mg of protein), expressed as a percent of control value (typically representing about 40 pmol of GTP). The graph contains results with $n = 6$ for experiments with protein from the same initial cell preparation.

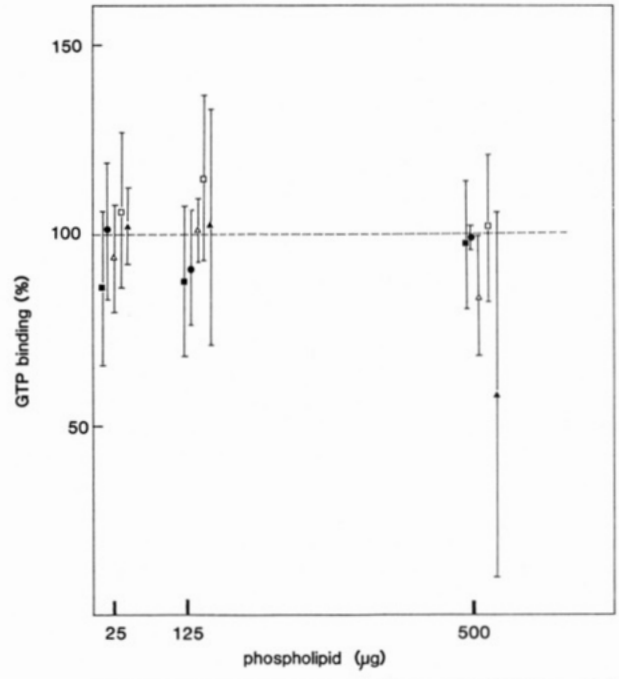

FIGURE 4: Effect of phospholipid composition on the binding of p21 (500 μL, containing about 1 mg of protein) with GTP. Results are expressed as percent of control values, typically representing about 40 pmol of GTP. In this experiment, the lipid coextracted with crude p21 from *E. coli* (see Figure 2, lane 2) was progressively displaced by the addition of increasing quantities of specific phospholipid species. Dimyristoylphosphatidylcholine [DMPC (●)], dipalmitoylphosphatidylcholine [DPPC (■)], and dioleylphospahtidylcholine [DOPC (□)], were used to alter the membrane thickness, having acyl chains of 14, 16, and 18 carbons, respectively; the unsaturated species (DOPC) would also increase membrane fluidity. The significance of the head group was probed by use of dipalmitoylphosphatidylethanolamine [DPPE (△)] and the negatively charged dipalmitoylphosphatidylserine [DPPS (▲)].

control values, to allow the incorporation of data from several different experiments. The alteration of membrane thickness by the species DMPC, DPPC, and DOPC (having 14, 16, and 18 carbons in their acyl chains, respectively) did not appear to affect GTP binding. The unsaturated species DOPC would, in addition, increase membrane fluidity. The significance of the phospholipid head group was investigated by use of DPPE and DPPS in addition to DPPC. Although no significant alteration was found in the affinity of p21 for GTP with any

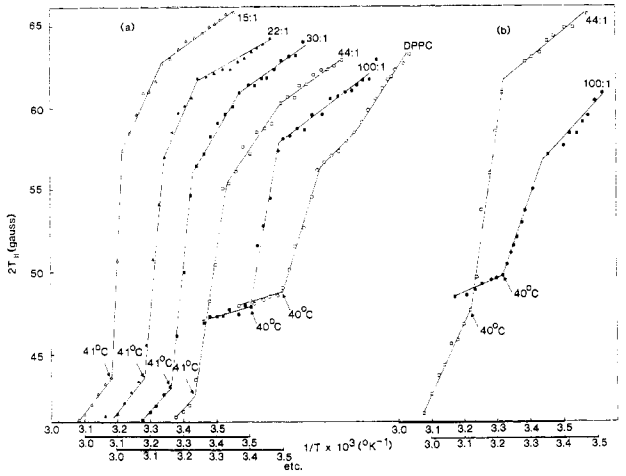

FIGURE 5: Plots of membrane fluidity (which is a function of the $2T_{\parallel}$ ESR splitting of 5-doxylstearate) vs $1/T$, for increasing proportions of p21 in synthetic vesicles of DPPC. The p21 was purified by two methods: (a) sonication of crude p21 (Figure 1, lane 2) successively with 0.5% octyl glucoside (3 times), 0.5% cholate (3 times), and 30 mM Tris-HCl, pH 7 (3 times), retaining the pellet from centrifugation at 12000*g* throughout; (b) extraction of crude p21 with chloroform–methanol (1:1). Preparation b gave similar results to those obtained with p21 in which no such delipification had been carried out (preparation a); this supports the assumption that the perturbation of the bilayer is indeed due to the effects of the p21 protein, rather than to contamination with associated cell lipid or detergents used in p21 extraction.

of these lipids, it was noted that DPPS produced GTP binding results that had a much larger standard deviation than those of the other lipid species (even though n was increased to 8 for results with this lipid; $n = 6$ for other lipids). The direct interference of the phospholipids with the binding assay was excluded as a possibility by the use of suitable controls.

Electron Spin Resonance. Figure 5 contains plots derived from ESR spectra of synthetic vesicles of DPPC at various protein to phospholipid ratios. The parameter $2T_{\parallel}$ gives an arbitrary measure of spin-label fluidity. The plots of the experiments with the p21 extracted under nondenaturing conditions indicate that the *ras* gene product p21 has a clear influence on membrane kinetics. A pronounced depression of the sharpness of the break at approximately 40 °C was noted at phospholipid to protein ratios below 44:1. With 100 lipids per p21 molecule, the change in gradient is far more pronounced at this break temperature. These results very closely match work obtained by previous workers for the Mg^{2+}-dependent Ca^{2+}-ATPase of sarcoplasmic reticulum (Hesketh et al., 1976). However, a major difference exists in our results for p21; even at the highest protein concentration tested, there was a break at approximately 40 °C. In Ca^{2+}-ATPase, below a phospholipid to protein ratio of about 30:1 no such transition is evident, as a phospholipid "annulus" appears to exist, intimately bound to the protein (Hesketh et al., 1976; Warren et al., 1975; Lentz et al., 1985). Thus in our study it appears that no such annulus is found.

Discussion

Our principal aim in this work was to determine the importance of the lipid environment for the function of p21 and therefore to investigate the nature of the association of p21 with the membrane. The separation of the protein components of the initial p21 preparation indicated that this preparation contained both multimolecular aggregates of protein (M_r >600K) and also individual protein molecules. Both fractions that contained p21 were active for GTP binding and contained associated phospholipid when analyzed by nondenaturing polyacrylamide electrophoresis and stained for lipid (unpublished data). Hence, both forms of p21 presumably exist with small amounts of associated phospholipid and detergent, and therefore have similarities with other forms of solubilized membrane protein (Duran & Cabib, 1978; Montgomery et al., 1984).

It was notable that our preparations of p21 were only sparingly soluble in water, even upon the addition of detergent such as cholate, octyl glucoside, or digitonin. Indeed, sonication with these solubilizing agents resulted in a dramatic improvement in the purity of the p21 retained in the pellets (Figure 2), indicating the hydrophobic nature of this protein. More significantly, after p21 was dissolved in 8 M urea, dialysis against dilute aqueous buffers maintained the protein in solution. Although these solutions retained GTP binding ability, there can be no doubt that important structural changes must have taken place to the p21 molecule. It is worth noting that all our other studies utilized p21 that had not been solubilized by denaturants.

The addition of cholate to the p21 preparation resulted in an increase in GTP binding at low detergent concentrations and a steady tail off of activity with increasing addition. This behavior is different to observations from similar experiments with transmembrane proteins (Montgomery & Gooday, 1985; Hesketh et al., 1976) where the delipification of the annular lipid surrounding such molecules leads to the irreversible denaturation of the enzyme, which appears as a rapid loss of enzymic activity above a critical concentration of added detergent. Retention of GTP affinity upon delipification certainly indicates that major structural changes to the binding site do not occur, although GTPase activity was not investigated directly.

The affinity of p21 for GTP was not altered by the modification of the lipid associated with the protein. No significant alteration was found for any of the lipid species tested. The thickness of the bilayer resulting from the acyl chain length of the phospholipid has previously been shown to affect the activity of transmembrane proteins (Duran & Cabib, 1978; Montgomery & Gooday, 1985). Phospholipids with various head groups were also incorporated in to this study as it was thought that this region of the molecule may have been important for p21 attachment to the inner surface of the membrane (Willingham et al., 1980).

The plots of the data from the ESR spectra of synthetic vesicles of DPPC at various p21 to phospholipid molar ratios (Figure 5) indicate a clear interaction of the *ras* gene product with the membrane. The function $2T_{\parallel}$ was used as a measure of the spin-label fluidity within the bilayer. A clear difference is noted between phospholipid:protein ratios of 100:1 and 44:1, and this indicates that the perturbation of the bilayer by p21 extends to somewhere between 44 and 100 phospholipids around the protein. This magnitude of membrane disruption is marginally smaller than that of the Ca^{2+}-ATPase of sarcoplasmic reticulum, which appears to be roughly 130–170 phospholipids (Lentz et al., 1985). This is probably due to either the p21 being a fundamentally much smaller protein or the p21 embedding itself less deeply in the membrane. The most interesting difference between these two proteins must be that even at the highest p21:phospholipid ratio employed a break in the plot gradient was observed at approximately 40 °C. The study of Hesketh et al. (1976) indicated that at below about 30 phospholipids per ATPase this transition was lost, as an intimately bound annulus of this number of lipids appears to surround the protein. Therefore, the interaction observed in our study suggests that the p21 interacts with the

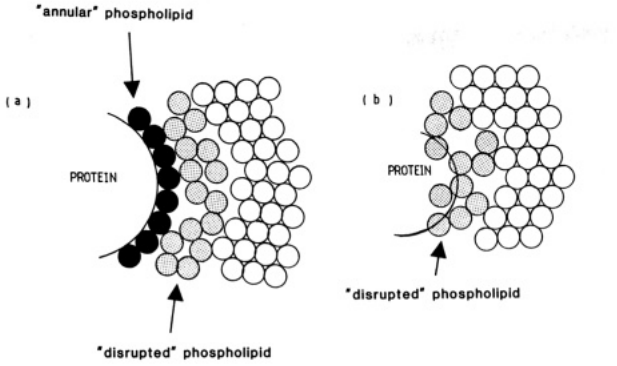

FIGURE 6: Diagrammatic representation of (a) the assumed interaction of a transmembrane protein with its surrounding phospholipid and (b) our proposed model of the association of p21 with the membrane.

cell membrane in a way clearly discernible from that of previously studied transmembrane proteins, having no annular lipid and perhaps disrupting a smaller population of proximal lipid (Figure 6). This interpretation supports the interpretation by Willingham et al. (1980) that the p21 is localized at the inside of the plasma membrane and that failure to detect p21 on the outside of the membrane is indeed due to the site of incorporation of the protein within the membrane.

Figure 5b contains a plot of the results obtained with a p21 preparation washed with chloroform–methanol (1:1 v/v) and indicates a similar result to that obtained with the "native" protein. Although this protein is irreversibly denatured by the addition of these organic solvents (unpublished results), it is presented as a control and supports the assumption that the membrane perturbation is indeed due to the p21 protein and is not due to hydrophobic molecules with nonspecific affinities for the membrane (nor detergent contaminants) as these molecules would have been removed by this, and prior, extraction steps.

In addition, the apparent differences of p21 proteins produced in *E. coli* found by groups working with different clones require comment. Gross et al. (1985) reported the production of a p21 protein which is soluble and discussed the apparent difference with the pJCL33 plasmid of Lacal et al. (1984) currently used in our research. The ability of the p21 protein produced from the pJCL33 plasmid to transform mammalian cells has been established (Lacal et al., 1986) and hence supports the assumption that this is a native *ras* protein, folded into a correct functional configuration. In addition, the ESR results in our study were obtained from such a p21 protein which had at no stage been exposed to denaturing chemicals such as urea.

Posttranslational addition of lipid to p21 is known to occur in mammalian cells. This modification is by a thioester bond to a cysteine near the carboxy terminus (Willumsen et al., 1984). It has also been suggested that this acylation is important for p21 attachment to the membrane in other experimental systems (Bartholomew et al., 1982). The hydrophobicity of the c-Ha *ras* used in this study was highlighted by its insolubility and the presence of copurified phospholipid in extraction and purification steps, even though the absence of any acylation of the p21 had been verified (unpublished results). We can be certain therefore that p21 can associate with the membrane, even without the modification of the Cys^{186}. The significance of this acylation may therefore be to accentuate the membrane affinity already present in p21 or to aid the interaction with receptor/effector molecules. This may be important in p21 attachment as large differences exist in the family of *ras* species, for example, the proportion of charged lysine groups the carboxy regions contain (Gatica et al., 1987; Lowe et al., 1987). Thus, the exact nature of the membrane associations of these proteins is still unclear, but an inherent affinity of the c-Ha *ras* p21 for the membrane unlike that of known transmembrane proteins is an undoubted feature of this molecule.

Registry No. GTP, 86-01-1; DMPC, 13699-48-4; DPPC, 2644-64-6; DOPC, 10015-85-7; DPPE, 3026-45-7; DPPS, 3036-82-6.

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