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Dynamic fatty acylation of p21$^{N\text{-}ras}$

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To study the acylation of p21$^{N\text{-}ras}$ with palmitic acid we have used cells which express the human N-*ras* gene to high levels under control of the steroid-inducible MMTV–LTR promoter. Addition of [^{3}H]palmitate to these cells resulted in detectable incorporation of label into p21$^{N\text{-}ras}$ within 5 min, which continued linearly for 30–60 min. Inhibition of protein synthesis for up to 24 h before addition of [^{3}H]palmitate had no effect on acylation of p21$^{N\text{-}ras}$, suggesting that this can occur as a late post-translational event. Acylated p21$^{N\text{-}ras}$ with a high SDS–PAGE mobility is found only in the membrane fraction, whereas ~50% of the [^{35}S]methionine-labelled p21$^{N\text{-}ras}$ is cytoplasmic and has a lower mobility. Conversion of the acylated high mobility form to a deacylated form of slightly lower mobility can be achieved with neutral hydroxylamine, which is known to cleave thioesters. This treatment also results in partial removal of p21$^{N\text{-}ras}$ from the membranes. A remarkably high rate of turnover of the palmitate moiety can be demonstrated by pulse–chase studies ($t_{1/2}$ ~20 min in serum-containing medium) which cannot be attributed to protein degradation. The data suggest an active acylation–deacylation cycle for p21$^{N\text{-}ras}$, which may be involved in its proposed function as a signal transducing protein.

Key words: ras/fatty acid/turnover

Introduction

Members of the mammalian *ras* oncogene family, N-*ras*, c-Ha-*ras1* and c-Ki-*ras2* (Marshall, 1986) each encode 21 000 relative molecular mass proteins, p21^{ras}, which are associated with the inner face of the plasma membrane (Willingham *et al.*, 1983). These proteins are capable of binding guanine nucleotides (Shih *et al.*, 1980) and exhibit GTPase activity (McGrath *et al.*, 1984). Sequence homology between the p21^{ras} proteins and the α-subunits of several G-proteins involved in signal transduction has led to the proposition that these proteins have analogous functions (Hurley *et al.*, 1984b). This proposal has found recent support in the studies of Wakelam *et al.* (1986), which showed that in NIH–3T3 fibroblasts expressing elevated levels of normal p21$^{N\text{-}ras}$ there is increased coupling between some growth factor receptors and inositol phospholipid turnover. Furthermore cells transformed by mutant p21^{ras} have elevated basal levels of inositol phospholipid turnover (Fleischman *et al.*, 1986; Wolfman and Macara, 1987; Wakelam *et al.*, in preparation).

p21^{ras} proteins are acylated with palmitic acid at a cysteine residue near the C terminus (Cys-186). This modification may contribute to the SDS–PAGE mobility change observed during pulse–chase experiments of newly synthesized p21^{ras} (Chen *et al.*, 1985; Buss and Sefton, 1986). Addition of palmitate to Cys-186 appears to be essential for membrane localization and transforming ability of the v-Ha-*ras* oncogene product (Willumsen *et al.*, 1984). Paradoxically microinjected p21^{ras} protein purified from *Escherichia coli* expression systems can induce many of the phenotypic events associated with transformation by *ras* genes (Feramisco *et al.*, 1984; Stacey and Kung, 1984; Bar-Sagi and Feramisco, 1986) despite the inability of the bacterial cell to acylate the protein (Chen *et al.*, 1985). Taken together these observations suggest that p21^{ras} must be capable of being acylated after microinjection. Using a cell line T15 which overexpresses the p21$^{N\text{-}ras}$ protein up to 50-fold under the control of the steroid-inducible MMTV–LTR promoter (McKay *et al.*, 1986) we show here that p21$^{N\text{-}ras}$ is indeed capable of being acylated uncoupled from translation and that removal of the fatty acid causes decreased membrane binding and reduces SDS–PAGE mobility. Strikingly the turnover of the palmitate moiety is very rapid compared with the lifetime of the protein in serum-containing medium. These data suggest a role for a dynamic acylation–deacylation cycle during p21$^{N\text{-}ras}$ function.

Results

Elevated level of p21$^{N\text{-}ras}$ in T15$^+$ cells

Addition of dexamethasone to the T15 cell line results in the overexpression of normal p21$^{N\text{-}ras}$ such that it accounts for up to 1% of the membrane protein (McKay *et al.*, 1986). Figure 1A (lanes 1–3) shows the increase in p21$^{N\text{-}ras}$ expression induced by growth in 2 μM dexamethasone, the concentration used throughout this study. This high level of expression greatly facilitates the study of p21$^{N\text{-}ras}$ acylation, since the endogenous protein is only detected with low sensitivity using [^{3}H]palmitic acid labelling (Figure 1A, compare lanes 4 and 6). Thus using induced T15 cells (T15$^+$) we were able to determine some of the properties of the acylation event.

Figure 1B demonstrates that p21$^{N\text{-}ras}$ in T15$^+$ cells induced with dexamethasone becomes one of the major palmitoylated proteins (lane 1), the identity of p21$^{N\text{-}ras}$ having been confirmed by immunoprecipitation of the same lysates with monoclonal antibody Y13-259 (Furth *et al.*, 1982, data not shown). Previous work from this laboratory and others has shown that palmitoylation of the bulk of cellular proteins is greatly reduced by inhibitors of protein synthesis (Magee and Courtneidge, 1985; McIlhinney *et al.*, 1985; Olsen *et al.*, 1985). Although inhibition (>95%) of protein synthesis by emetine during labelling strikingly depresses acylation of most cellular proteins, the acylation of p21$^{N\text{-}ras}$ is completely unaffected (Figure 1B, lane 2). Similar results are obtained if [^{3}H]palmitate is added as much as 24 h after blockage of protein synthesis (data not shown). In addition cerulenin, which has been shown to be an inhibitor of protein acylation (Schlesinger and Malfer, 1982; Kotwal and Ghosh, 1984), is without effect on p21$^{N\text{-}ras}$ acylation at concentrations where it does not inhibit protein synthesis (Magee and Gutier-

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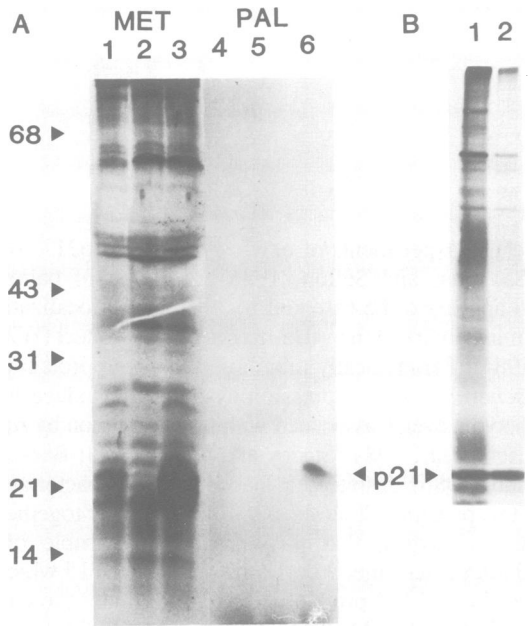

Fig. 1. Expression of acylated p21$^{N\text{-}ras}$ in T15 cells and effect of emetine on acylation (**A**) Immunoprecipitation showing the dexamethasone-induced increase in expression of p21$^{N\text{-}ras}$ in T15 cells. Cells were grown in 50 mm dishes until subconfluent. Dexamethasone (2 μM) was added to two dishes (T15$^+$) and 16 h later the cells were labelled for 3.5 h with 200 μCi [^{3}H]palmitate in 2 ml FA medium (**lanes 4–6**) or 100 μCi [^{35}S]methionine in 2 ml methionine-free medium (**lanes 1–3**). **Lanes 1** and **4**: uninduced cells (T15$^-$) immunoprecipitated with Y13-259 monoclonal anti-p21^{ras} antibody; **lanes 2** and **5**: induced cells (T15$^+$) immunoprecipitated with non-immune rat serum; **lanes 3** and **6**: T15$^+$ cells immunoprecipitated with Y13-259. (**B**) Total palmitate-labelled proteins of T15$^+$ cells treated (**lane 2**) or untreated (**lane 1**) with the protein synthesis inhibitor emetine. Cells were labelled for 4 h with 75 μCi/ml [^{3}H]palmitate either with (**lane 2**) or without (**lane 1**) a 1 h pre-incubation with 10 μM emetine, washed twice with ice-cold PBS, lysed directly in Laemmli loading buffer plus DTT and electrophoresed on a 12.5% gel.

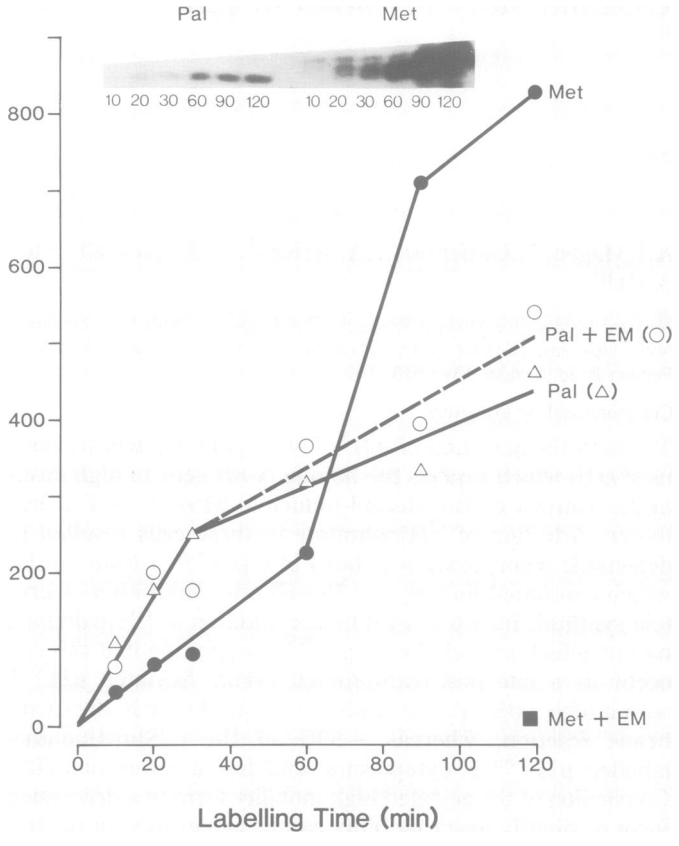

Fig. 2. Kinetics of fatty acid incorporation into p21$^{N\text{-}ras}$. T15$^+$ cells in 35 mm × 6 well Costar dishes were pre-incubated in FA medium or methionine-free medium for 30 min in the presence or absence of 10 μM emetine (EM) and then labelled with 33 μCi/ml [^{35}S]methionine (MET) or 133 μCi/ml [^{3}H]palmitate (PAL) for the indicated times. At each time point cells were washed, lysed and immunoprecipitated with Y13-259 monoclonal antibody followed by SDS–PAGE and fluorography. The insert shows the fluorograph of the immunoprecipitated p21$^{N\text{-}ras}$. A single experiment is shown, but similar results were obtained on two other occasions.

rez, unpublished data). Interestingly some other cellular acyl proteins show unimpaired labelling after inhibition of protein synthesis (Figure 1B; Magee and Courtneidge, 1985) and may constitute a separate class of palmitoylated proteins.

Post-translational acylation of p21$^{N\text{-}ras}$

If p21$^{N\text{-}ras}$ can be acylated efficiently many hours after synthesis it is possible that this modification might be a dynamic event involving cycles of acylation and deacylation. In this case continuous incorporation of [^{3}H]palmitate should lead to a steady-state level of labelling of p21$^{N\text{-}ras}$ as has been shown for the acylation of erythrocyte ankyrin (Staufenbiel, 1987). Figure 2 shows the results of such an experiment where [^{3}H]palmitate or [^{35}S]methionine have been continuously incorporated into T15$^+$ cells for up to 120 min. Methionine is linearly incorporated into immunoprecipitated p21$^{N\text{-}ras}$ during this time, whereas the rate of labelling with palmitic acid slows down after ~30 min, consistent with a rapid turnover of fatty acid on p21$^{N\text{-}ras}$. Ten micromolar emetine did not change the kinetics of [^{3}H]palmitate incorporation despite inhibiting [^{35}S]methionine incorporation by >95%, showing that [^{3}H]palmitate was being incorporated into previously synthesized p21$^{N\text{-}ras}$.

Turnover of fatty acid moiety of p21$^{N\text{-}ras}$

Turnover has been observed directly by pulse–chase labelling of T15$^+$ cells (Figure 3). The top panel of Figure 3A shows the electrophoretic analysis of immunoprecipitates of [^{35}S]methionine-labelled p21$^{N\text{-}ras}$ after a 2 h pulse followed by chases of up to 3 days. It is clear that p21$^{N\text{-}ras}$ is quite stable under these conditions, with a half-life of the order of 1 day (Figure 3B) in agreement with studies of Ulsh and Shih (1984). In contrast initial experiments showed that the fatty acyl moiety of p21$^{N\text{-}ras}$ turned over rapidly and was completely lost within 1 day. Short time-course pulse–chase experiments with [^{3}H]palmitate show that p21$^{N\text{-}ras}$-associated palmitate is turning over with a half-life of ~20 min (Figure 3A, lower panel and Figure 3B); this is consistent with a dynamic acylation–deacylation cycle. A similar fatty acid turnover rate was seen with a cell line over-expressing the normal Ha-*ras* proto-oncogene product (data not shown). About 10% of the label derived from [^{3}H]palmitate apparently turns over much more slowly (Figure 3B); this may be due to reincorporation of label. A similar phenomenon has been observed for the transferrin receptor (Omary and Trowbridge, 1981).

Membrane association of acylated p21$^{N\text{-}ras}$

It has been suggested that acylation of proteins in general and p21^{ras} in particular may mediate their association with the plasma membrane (Magee and Schlesinger, 1982; Willumsen *et al.*, 1984). Direct evidence for this has been lacking however. Figure 4A confirms that in T15$^+$ cells at steady-state the p21$^{N\text{-}ras}$ is distributed between the cytosol (lane 1) and the membrane fraction (lane 2). The cytosolic form of p21$^{N\text{-}ras}$ has a

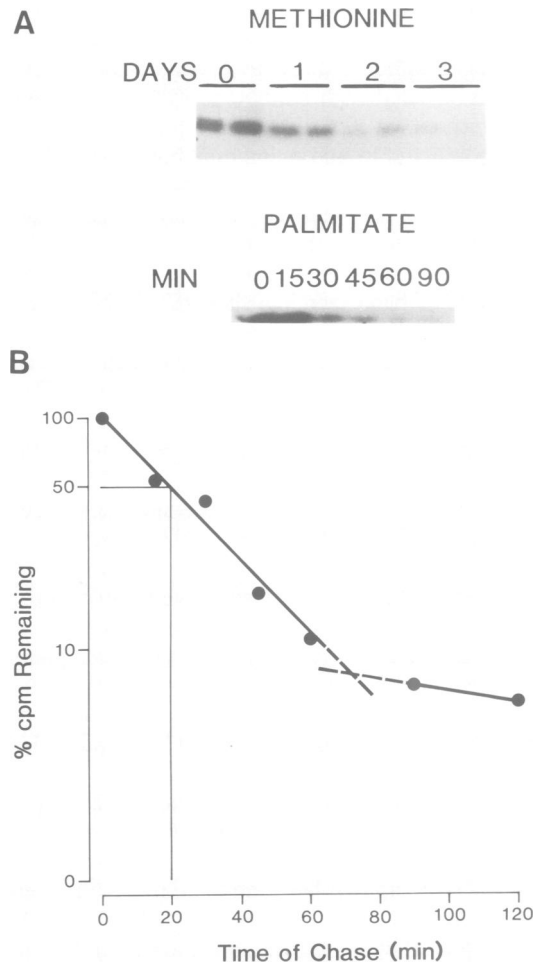

Fig. 3. Turnover of fatty acid on p21$^{N\text{-}ras}$. **(A)** 35 mm dishes of T15^{+} cells were labelled in duplicate with 50 μCi/ml [^{35}S]methionine in methionine-free medium for 2 h, then chased in medium containing 10 times the normal methionine content for the indicated number of days. Lower panel: T15^{+} cells were pre-incubated in 1 ml FA medium for 30 min, labelled for 5 min with 200 μCi [^{3}H]palmitate and chased for the indicated time in min with fresh FA medium. Cells were washed, lysed, immunoprecipitated and analysed by SDS−PAGE and fluorography. **(B)** The fluorograph of [^{3}H]palmitate-labelled p21$^{N\text{-}ras}$ was scanned and the intensity of the image plotted versus time of chase. $t_{1/2}$ for fatty acid turnover is estimated from this and at least eight other experiments to be ~20 min.

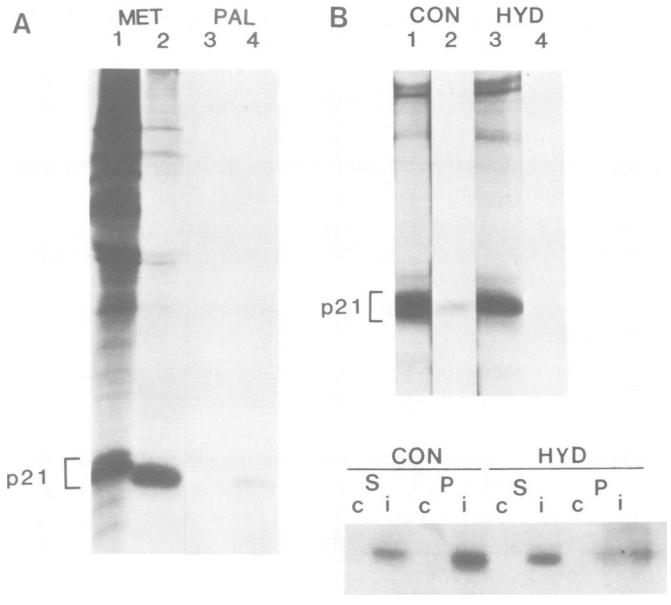

Fig. 4. Membrane association of palmitoylated p21$^{N\text{-}ras}$ and the effect of hydroxylamine. T15^{+} cells in 150 mm dishes were labelled for 4 h with 30 μCi/ml [^{35}S]methionine in low methionine medium or 100 μCi/ml [^{3}H]palmitate in FA medium (8 ml total). Cells were lysed and fractionated as described in Materials and methods. **(A)** Shows the distribution of [^{35}S]-methionine-labelled (**lanes 1** and **2**) and [^{3}H]palmitate-labelled (**lanes 3** and **4**) p21$^{N\text{-}ras}$ between equivalent amounts of soluble (**lanes 1** and **3**) and membrane-bound (**lanes 2** and **4**) fractions. **(B) Upper panel:** deacylation of p21$^{N\text{-}ras}$ with hydroxylamine ([^{3}H]palmitate-labelled; compare **lanes 2** and **4**) without loss of protein ([^{35}S]methionine-labelled; **lanes 1** and **3**). Aliquots of the membrane fraction were treated for 1 h at 23°C with 1 M Tris-Cl, pH 8 (**CON**) or 1 M hydroxylamine, pH 8 (**HYD**), lysed, immunoprecipitated and analysed by SDS−PAGE and fluorography. **Lower panel:** partial removal of p21$^{N\text{-}ras}$ from the membrane-bound fraction after deacylation with hydroxylamine (**HYD**), and the associated SDS−PAGE shift. [^{35}S]-methionine-labelled membranes isolated as above were similarly treated with Tris or hydroxylamine and re-isolated by centrifugation. The supernatant (**S**) and pellet (**P**) were analysed for the presence of p21$^{N\text{-}ras}$ by immunoprecipitation (**c**, no Y13-259 antibody; **i**, Y13-259 precipitation), SDS−PAGE and fluorography.

slower migration on SDS−PAGE, than the membrane-bound form. No [^{3}H]palmitate is incorporated into cytosolic p21$^{N\text{-}ras}$, whereas the faster-migrating membrane-bound form is acylated. The relative proportions of membrane-bound and cytosolic p21$^{N\text{-}ras}$ do not change significantly over a 20-fold range of total p21$^{N\text{-}ras}$ concentration (data not shown). Treatment with neutral hydroxylamine has been used as a diagnostic test for ester-linked fatty acid and as a method for gentle removal of fatty acid from protein without denaturation (Omary and Trowbridge, 1981; Magee *et al.*, 1984; Berger and Schmidt, 1984). Figure 4B (upper panel) shows that hydroxylamine treatment efficiently removes [^{3}H]palmitate from immunoprecipitated p21$^{N\text{-}ras}$ (Figure 4A, lanes 2 and 4) without any loss of [^{35}S]-methionine-labelled p21$^{N\text{-}ras}$ (Figure 4A, lanes 1 and 3).

We have also tested the effect of hydroxylamine deacylation of p21$^{N\text{-}ras}$ on its membrane association and electrophoretic mobility. Membranes containing [^{35}S]methionine-labelled p21$^{N\text{-}ras}$ were prepared from T15^{+} cells, treated with hydroxylamine or mock-treated and the membranes re-isolated by centrifugation. The distribution of p21$^{N\text{-}ras}$ between the pellet (P) and the supernatant (S) was assessed by immunoprecipitation. Figure 4B (lower panel) shows that while membranes mock-treated with Tris buffer retained most of the [^{35}S]methionine-labelled p21$^{N\text{-}ras}$, the hydroxylamine-treated membranes had been depleted of p21$^{N\text{-}ras}$, which was released into the supernatant. This provides evidence for the direct involvement of covalently-bound palmitate in the membrane association of p21$^{N\text{-}ras}$. In several experiments quantitative release of p21$^{N\text{-}ras}$ could not be achieved (usually ~50%) despite apparently complete deacylation. This was true even after sonication of the membranes or treatment with 0.1 M Na_2CO_3, pH 11 (data not shown).

Post-translational processing of p21$^{N\text{-}ras}$

The characteristic post-translational SDS−PAGE mobility changes associated with p21$^{N\text{-}ras}$ maturation can be seen in the [^{35}S]methionine pulse-labelling experiments shown in Figure 2. At short labelling times (10 and 20 min) a slow migrating third form of p21$^{N\text{-}ras}$ can be seen which decreases in prominence at the longer labelling times. In pulse−chase experiments with [^{35}S]methionine this slow p21$^{N\text{-}ras}$ (pp21), rapidly chases into the two faster migrating forms seen during steady-state labelling, while a single acylated species corresponding to the fastest migrating form is seen (data not shown). In addition to the ef-

fect of hydroxylamine on the deacylation and membrane association of $p21^{N\text{-}ras}$, it also caused a slight decrease in the mobility of the protein (Figure 4B, lower panel), consistent with a direct role of acylation in the SDS−PAGE mobility increase associated with maturation of $p21^{N\text{-}ras}$ (Willumsen *et al.*, 1984; Buss and Sefton, 1986). This decrease in mobility does not account totally for the mobility shift during maturation (i.e. pp21 conversion to p21) and cannot always be resolved (compare Figure 2B, upper and lower panels). Other modifications may also contribute to mobility changes.

Discussion

The major conclusion from this study is that acylation of $p21^{N\text{-}ras}$ is a dynamic event. This is demonstrated by the rapid approach to a steady-state level of acylation (Figure 2) even in the absence of protein synthesis and by the short half-life (~20 min) of the fatty acid moiety (Figure 3). These data imply a rapid acylation−deacylation cycle of $p21^{N\text{-}ras}$ *in vivo*. Turnover of fatty acid moieties during the lifetime of a protein has also been observed for the transferring receptor (Omary and Trowbridge, 1981) and ankyrin (Staufenbiel, 1987), although in neither case was it as rapid as reported here for $p21^{N\text{-}ras}$.

It has been proposed that $p21^{ras}$ proteins are members of the family of G-proteins (Hurley *et al.*, 1984b) whose function is to mediate the interaction between cell surface receptors and effector signalling systems associated with the inner face of the plasma membrane. Wakelam *et al.* (1986) have recently demonstrated in $T15^+$ cells that $p21^{N\text{-}ras}$ is capable of enhancing coupling between certain growth factor receptors and inositol phospholipid turnover. It will be interesting to see whether the acylation−deacylation cycle reported here has a role in the functional cycle of $p21^{N\text{-}ras}$. Analogous experiments to those of Wakelam *et al.* (1986) are under way using defined growth factors, to assess their effect on $p21^{N\text{-}ras}$ palmitate turnover.

Endogenously synthesized $p21^{N\text{-}ras}$ can be acylated post-translationally (Figure 1B; Figure 2), which may explain the ability of unacylated bacterially-expressed $p21^{ras}$ to function after microinjection (Feramisco *et al.*, 1984; Stacey and Kung, 1984; Bar-Sagi and Feramisco, 1986), despite evidence that acylation is absolutely required for function (Willumsen *et al.*, 1984). $p21^{N\text{-}ras}$ acylation is resistant to cerulenin (not shown) as well as to protein synthesis inhibitors (Figures1B and 2), despite the sensitivity of most other cellular acyl proteins to these agents. This taken together with the knowledge that most protein palmitoylation occurs in the early Golgi complex (Schmidt and Schlesinger, 1980; Dunphy *et al.*, 1981) suggests that the enzymatic machinery for acylation of $p21^{N\text{-}ras}$ may differ from that of the bulk of cellular palmitoylated proteins. Alternatively there may be two pools of acylation substrates, one freely accessible to the acylation machinery (e.g. $p21^{N\text{-}ras}$) and the other inaccessible due to intracellular localization.

Fatty acylation of $p21^{N\text{-}ras}$ could affect its function in a number of ways. Acylation of proteins has been suggested to contribute to their association with membranes (Magee and Schlesinger, 1982; Schmidt, 1983), and this has been confirmed in the case of myristoylation of $pp60^{src}$ (Kamps *et al.*, 1985; Pellman *et al.*, 1985). The importance of acylation for membrane association of $p21^{ras}$ proteins has been elegantly demonstrated using mutants lacking the crucial Cys residue at position 186 (Willumsen *et al.*, 1984), which were unable to transform cells. Viral $p21^{ras}$ proteins without this acylation site failed to incorporate [^{3}H]palmitate, did not associate with membranes or undergo the characteristic increase in mobility on SDS−PAGE.

We present additional direct evidence here that acylation of $p21^{N\text{-}ras}$ is associated with membrane binding and with a shift from an intermediate SDS−PAGE mobility to a fast mobility. Both this mobility shift and membrane binding can be reversed by deacylation with hydroxylamine, although removal from the membrane was not quantitative. This may indicate that $p21^{N\text{-}ras}$ has other associations with the membrane or with proteins, which are not fatty-acid mediated. The mobility shift from the slowest-migrating $p21^{N\text{-}ras}$ band seen in a short pulse-label (pp21; Figure 2) to the cytosolic band of intermediate mobility occurs rapidly (~15 min) in these cells and does not correlate with acylation, in agreement with Chen *et al.* (1985). The nature of this mobility shift remains obscure.

Sequence comparison between $p21^{ras}$ proteins and the α-subunits of all known G-proteins has revealed homologous conserved regions believed to be involved in guanine nucleotide binding (residues 109−120 of $p21^{Ha\text{-}ras}$) and GTPase activity (residues 6−20 of $p21^{Ha\text{-}ras}$). $p21^{ras}$ proteins have an additional region of homology to the *Pertussis* toxin-sensitive G-protein α-subunits (α_1, α_0, Tα) but not found in the α_s-subunit, consisting of the C-terminal sequence Cys-A-A-X, where A is an aliphatic amino acid and X is any amino acid (Tanabe *et al.*, 1985; Itoh *et al.*, 1986). At least one γ-subunit, from bovine retinal transducin, also contains a similar sequence at its C terminus, despite the lack of any other homology to $p21^{ras}$ (Hurley *et al.*, 1984a). Since the corresponding cysteine residue in $p21^{ras}$ proteins is acylated, it has been suggested that a similar modification might occur on the G-protein α-subunits (Lochrie *et al.*, 1985). This region of the α-subunits is believed to be involved in interaction with receptors (Cote *et al.*, 1984; Bourne, 1986). It is noteworthy that this conserved cysteine residue is the site of ADP ribosylation by *Pertussis* toxin, which inactivates the α_i subunit, suggesting a crucial role for this region in G-protein function, possibly involving fatty acylation (Von Dop *et al.*, 1984; Ui *et al.*, 1984; West *et al.*, 1985). We have looked for an effect of *Pertussis* toxin on $p21^{N\text{-}ras}$ in $T15^+$ cells but have found no evidence for ADP ribosylation of $p21^{N\text{-}ras}$ despite extensive modification of an M_r ~40-K substrate, nor for any effect of *Pertussis* toxin treatment on acylation of $p21^{N\text{-}ras}$ or turnover of fatty acid (A.I.Magee, L.Gutierrez and L.Mahadevan, unpublished data). Thus it seems that depsite sequence similarities in the acylation site of $p21^{N\text{-}ras}$ and the *Pertussis* toxin ADP ribosylation site in *Pertussis*-sensitive α-subunits there must be differences which contribute to these two properties.

In $T15^+$ cells we consistently observe a significant pool (~50%) of the $p21^{N\text{-}ras}$ in a non-acylated soluble form (Figure 4A). This does not seem to be due to saturation of membrane binding sites, since varying the amount of $p21^{N\text{-}ras}$ protein by changing the dexamethasone concentration in the medium over a 20-fold range did not change these proportions. Other workers have also observed a cytosolic pool of $p21^{ras}$ proteins (Myrdahl and Auersberg, 1985). Quantitative binding of $p21^{ras}$ to membranes has been found only with transforming $p21^{ras}$ products, suggesting that this may be a reflection of the activation process (Willumsen *et al.*, 1984; Shih *et al.*, 1982). It seems likely that *in vivo* acylation may act transiently during signalling to modify the interaction of $p21^{N\text{-}ras}$ with the membrane or with other components of the signal-transducing apparatus without actual dissociation from the membrane.

Materials and methods

Cell culture and labelling

T15 cells (McKay *et al.*, 1986) were cultured in Dulbecco's modified Eagle's medium plus 10% (v/v) newborn calf serum in the absence ($T15^-$) or presence ($T15^+$) of 2 μM dexamethasone (Sigma Chemical Co., Dorset, UK). Cells were labelled for the times indicated in the figure legends using [^{35}S]methionine (>1000 Ci/mmol, Amersham International plc, Bucks, UK) in methionine-free medium or in medium containing one tenth of the normal methionine concentration (low methionine medium), or [9,10-^{3}H]palmitic acid (Amersham, 50 Ci/mmol) in medium supplemented with 5 mM Na pyruvate (FA medium).

Immunoprecipitation and SDS–PAGE

Cell monolayers were washed twice in ice-cold 10 mM Na phosphate-buffered saline (PBS) and lysed in RIPA buffer [20 mM Tris, 150 mM NaCl, 1 mM EDTA, 0.5% (v/v) Nonidet P40, 0.5% (w/v) Na deoxycholate, 0.1% (w/v) SDS, 1% (v/v) Trasylol (Sigma), 0.2 mM phenylmethylsulphonyl fluoride, pH 7.4]. After 10 min on ice the lysates were centrifuged at 12 000 *g* for 10 min and the supernatants immunoprecipitated with 5 μl normal rat serum or excess Y13-259 pan-*ras* rat monoclonal antibody (Furth *et al.*, 1982) followed by rabbit anti-rat-coated Protein A-Sepharose (Sigma). Immunoprecipitates were washed three times with RIPA buffer, once with 100 mM Tris–Cl, pH 6.8 and then dissolved in Laemmli loading buffer containing 100 mM dithiothreitol, followed by electrophoresis in a 12.5% SDS–polyacrylamide gel (Laemmli, 1970). Bands were detected by fluorography using pre-flashed Kodak XAR-5 film (Bonner and Laskey, 1974). Bands were quantitated by excision and scintillation counting or by scanning of the film using a Zeineh soft laser densitometer.

Membrane fractionation

Cells in 150 mm dishes were washed twice with ice-cold PBS, once with lysis buffer (10 mM Hepes, 1 mM $MgCl_2$, 1 mM EGTA, pH 7.4), scraped into lysis buffer and swollen on ice for 15 min. Cells were broken with 40 strokes of a tight-fitting Potter–Elvehjem homogenizer, and debris was removed by centrifugation at 3000 r.p.m. for 10 min in a bench centrifuge at 4°C. The post-nuclear supernatant was centrifuged for 30 min at 30 000 r.p.m. at 4°C in a Beckman SW55 rotor. The pelleted membranes were resuspended in PBS. For deacylation membranes were resuspended in 1 M Tris-Cl, pH 8 or freshly prepared 1 M hydroxylamine-Cl (BDH Chemicals Ltd., Essex, UK) titrated to pH 8 with NaOH, and incubated for 1 h at 23°C. Membranes were re-isolated by centrifugation as above.

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