Ras GTPases undergo post-translational modifications that govern their subcellular trafficking and localization. In particular, palmitoylation of the Golgi tags N-Ras and H-Ras for exocytotic transport and residency at the PM (plasma membrane). Following depalmitoylation, PM-Ras redistributes to all subcellular membranes causing an accumulation of palmitate-free Ras at endomembranes, including the Golgi and endoplasmic reticulum. Palmitoylation is unanimously regarded as a critical modification at the crossroads of Ras activity and trafficking control, but its precise relevance to native wild-type Ras function in growth factor signalling is unknown. We show in the present study by use of palmitoylation-deficient N-Ras mutants and via the analysis of palmitate content of agonist-activated GTP-loaded N-Ras that only palmitoylated N-Ras becomes activated by agonists. In line with an essential role of palmitoylation in Ras activation, dominant-negative RasS17N loses its blocking potency if rendered devoid of palmitoylation. Live-cell Ras–GTP imaging shows that N-Ras activation proceeds only at the PM, consistent with activated N-Ras–GTP being palmitoylated. Finally, palmitoylation-deficient N-Ras does not sustain EGF (epidermal growth factor) or serum-elicited mitogenic signalling, confirming that palmitoylation is essential for signal transduction by N-Ras. These findings document that N-Ras activation proceeds at the PM and suggest that depalmitoylation, by removing Ras from the PM, may contribute to the shutdown of Ras signalling.

Key words: endomembrane, location, palmitoylation, plasma membrane, Ras.

INTRODUCTION

Nascent K-Ras, N-Ras and H-Ras (collectively known as Ras) proteins are subjected to four sequential default post-translational modifications at their conserved, but distinct, C-termini. Following farnesylation at Cys186 (Cys185 in K-Ras4B), which causes loose and dynamic association of Ras proteins with the ER (endoplasmic reticulum), Golgi and/or other internal membranes (collectively referred to as endomembranes), the last three amino acids are removed and the C-terminal carboxyl group is methylated by specific enzymatic systems [1,2]. At this point H-Ras, N-Ras and the minor isoform K-Ras4A become palmitoylated at two or one C-terminal cysteine residues (Cys181 and Cys184 in H-Ras; Cys181 in N-Ras), probably at the this previously characterized ‘acylation–deacylation cycle’ [3,4], the palmitoylation status dictates subcellular trafficking and localization of Ras.

On the basis of the currently debated notion that Ras may deliver distinct signals from the PM compared with endomembranes [9,10], it has been proposed that the spatial segregation of Ras–GTP as governed by the palmitoylation–depalmitoylation cycle may provide a means to effectively increase and diversify the signalling output of Ras by propagating the Ras–GTP signal elicited at the PM towards endomembranes. However, whether and to what extent palmitoylation or its loss affect native Ras function in the context of growth factor signalling has proved difficult to investigate and is largely unknown. Also, information on the fractional or total amount of palmitate-free N- and H-Ras present at endomembranes at steady state or in growth-factor-stimulated cells is yet lacking owing largely to the fact that the labile nature of the peptidyl-S-palmitoyl thioester bond has complicated the biochemical analysis of Ras palmitoylation required to address these and other questions. In the present study, we have addressed the role of palmitoylation in N-Ras activation and signal transmission downstream of activated growth factor receptors. The present study was thus aimed at clarifying whether or not unpalmitoylated N-Ras does function as a signalling mediator in the context of growth factor signalling.
EXPERIMENTAL

Materials

[9,10-3H]Palmitic acid 30–60 Ci (1.11–2.22 TBq)/mmol was from Hartmann Analytic. PMA was purchased from Enzo. EGF (epidermal growth factor) was acquired from Peprotech. ATC (anhydrotrycetinylcine) was purchased from Fisher Scientific. The Golgi marker BODIPY® TR C5 Ceramide was from Molecular Probes. Anti-BrdU (bromodeoxyuridine) antibody proliferation kit was from GE Healthcare.

Plasmids

HA (haemagglutinin)–N-RasG12V, HA–N-RasC181S and HA–N-RasC181S/G12V were generated from murine HA–N-Ras in pCMV-HA [11] by standard point mutagenesis. Myc–H-RasS17N in pNRTIS-21 has been described previously [12]. Myc–H-RasS17N/C181S/C184S was generated by point mutagenesis of the former. Lentiviral transfer vectors coding for HA–N-Ras or HA–N-RasC181S were generated by subcloning an Xbal/Xbal cassette from the pCMV-HA plasmids described above into pCDH-CMV-MCS-EF1-Puro (System Biosciences) linearized with Xbal.

Cell lines, cell culture and generation of stable cell lines

COS-7 [A.T.C.C. (Manassas, VA, U.S.A)], MCF-7 cells (a gift from Joachim Clement, Department of Hematology and Oncology, University Hospital Jena, Jena, Germany) and H-Ras (−/−), N-Ras (−/−), K-Ras (−/−) and RERTnert (in short, K-Raslox/lox) MEFs (mouse embryonic fibroblasts) [a gift from Mariano Barbacid, Molecular Oncology Programme, Centro Nacional de Investigaciones Oncológicas (CNOI), Madrid, Spain] were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % heat-inactivated FBS. To induce the Rasless state in K-Raslox/lox MEFs, seeded on to collagen-I-coated glass coverslips, K-Raslox/lox MEFs, seeded on to collagen-I-coated glass coverslips, were treated with or without 4-OHT for 3 days and subjected to two consecutive infection rounds with freshly generated lentiviral particles on days 4 and 5. Cells were grown for a further 3 days in the presence or absence of 4-OHT, serum-starved overnight and processed as required.

Antibodies

The following antibodies were used against: Pan-Ras (Ab-4) (Merck), N-Ras (F155) (Santa Cruz Biotechnology), phospho-p44/42 MAPK (mitogen-activated protein kinase) (ERK1/2) (Thr202/Tyr204) (E10), ERK (extracellular-signal-regulated kinase), phospho-Akt (Ser473) (D9E), Akt and HA-tag (6E2) (Cell Signaling Technology), and RalA (BD Pharmingen).

Ras–GTP and Ral–GTP assays

Ras–GTP levels were determined by pulling out active Ras–GTP complexes from cell extracts using the RBD (Ras-binding domain) of the Ras effector c-Raf fused to GST (GST–RBD) as described previously [14]. Ras–GTP pull-down assays were performed in essentially the same way, but for a different lysis solution: 50 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM MgCl2, 1 % glycerol, 1 % NoniD P40, 100 μM GDP, protease inhibitors (1 mM PMSF, 1 mM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 mM Pefabloc®), supplemented with 25 μg/ml of the Ral-binding domain of RalBP1 fused to GST (GST–RalBD). GST–RBD and GST–RalBD proteins were expressed and purified by standard procedures from BL21DE3pLysS or AD202 bacteria respectively.

Lentiviral transduction of Rasless MEFs

K-Raslox/lox MEFs were treated with or without 4-OHT for 3 days and subjected to two consecutive infection rounds with freshly generated lentiviral particles on days 4 and 5. Cells were grown for a further 3 days in the presence or absence of 4-OHT, serum-starved overnight and processed as required.

Metabolic labelling with [3H]palmitic acid

COS-7 or MCF-7 cells in 60-mm-diameter dishes were transfected with plasmid DNA and deprived of serum as appropriate for each experiment. [9,10-3H]Palmitic acid (30–60 Ci/mmol, in ethanol) was concentrated by evaporation of ethanol using a constant stream of nitrogen and resuspension in DMEM. Cells were labelled at 4 h with 0.5 mCi of 3H-labelled palmitate in 1.5 ml of medium, challenged with growth factors, placed on ice and washed twice with cold PBS. Cells were lysed and processed for the isolation of active N-Ras–GTP complexes as described above. To preserve palmitoylation, samples were not boiled before electrophoresis, but instead incubated in loading buffer lacking reducing agents for 1 h at room temperature (24°C). Samples were then resolved via SDS/PAGE (12.5 % gel) at low amperage overnight in a cold-room and processed for Western blotting and fluorography. For the latter, gels were soaked in Amplify (GE Healthcare), dried and exposed to a film for 4 weeks.

Ras–GTP visualization in live cells

Ras–GTP visualization in live MCF-7 cells was performed exactly as described in [12] using the E3-R3(A/D) reporter for Ras–GTP [11].

BrdU incorporation

K-Raslox/lox MEFs, seeded on to collagen-I-coated glass coverslips, were treated with or without 4-OHT for 3 days and infected with lentiviral particles coding for N-Ras or N-RasC181S as described above. After 2 days the cells were deprived of serum for 48 h, followed by serum re-supplementation. At 18 h later the cells were administered 20 μM BrdU labelling reagent (GE Healthcare) for 2 h and processed further following the manufacturer’s instructions.

RESULTS

Palmitoylation is required for growth factor activation of N-Ras

To delineate the role of palmitoylation in Ras activation and signal transduction we chose to study N-Ras rather than H-Ras for two reasons: first, we observed that growth factors activated endogenous and ectopic N-Ras more strongly than H-Ras in COS-7 and HeLa cells [15], suggesting that N-Ras provided a more robust experimental readout. Moreover, H-Ras expression is low or undetectable in numerous cell types.

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Figure 1 Palmitoylation-deficient N-Ras is not activated by EGF or PMA

(A) Activation of endogenous N-Ras in COS-7 cells stimulated with 10 ng/ml EGF for various periods of time. Immunodetection was performed with anti-N-Ras antibody. (B) COS-7 cells transiently expressing HA–N-Ras or palmitoylation-deficient HA–N-RasC181S were deprived of serum and challenged with 10 ng/ml EGF for the indicated periods of time. Active Ras–GTP (Ras–GTP pull down, upper panel) and total Ras levels (lower panel) were determined as described in the Experimental section. (C) Quantification of N-Ras–GTP levels from (A) and (B). Resting N-Ras–GTP levels were set to 1 for each condition. Data are shown as means ± S.E.M. (n = 3). (D) Same as (A), except cells were stimulated with 100 nM PMA. (E) COS-7 cells expressing HA–N-Ras or palmitoylation-deficient HA–N-RasC181S were deprived of serum and challenged with 100 nM PMA for the indicated periods of time. Western detection was performed with anti-N-Ras. (F) Quantification of N-Ras–GTP levels from (D) and (E). Data are shown as means ± S.E.M. (n = 3). (G) COS-7 cells were transfected with mCherry-tagged N-Ras versions followed by determination of mCherry–N-Ras–GTP levels using anti-N-Ras antibody for immunodetection. End. Ras, endogenous Ras; wt, wild-type. Asterisks in (B) and (E) denote a degradation product of HA-tagged N-Ras. The asterisk in (G) marks a degradation product of GST–RBD. Molecular masses are indicated in kDa.

[11,15], rendering the analysis of native H-Ras yet more difficult. Secondly, the presence of two palmitoylation sites in H-Ras can generate ambiguous experimental outcomes as a result of their varying relative occupancy with palmitate, adding considerable complexity to experimental design and data interpretation [16,17]. EGF induces activation of all three Ras isoforms in a number of cell types including COS and HeLa cells (Figure 1A and results not shown) [15,18,19]. To learn whether palmitoylation was required for growth factor activation of N-Ras we employed the mutant N-RasC181S, which cannot be palmitoylated and, in consequence, distributes largely to endomembranes [4,12,20] (see below, Figures 4C and 4D). As shown in Figure 1(B) EGF induced robust activation of HA-tagged wild-type N-Ras in COS-7 cells with a time course comparable with that of native Ras. In contrast, GTP loading of palmitoylation-deficient HA–N-RasC181S was marginal in or absent from COS-7 cells (Figures 1B and 1C) and other tested cell lines including MCF-7, HeLa and Jurkat T-cells challenged via cross-linking of the T-cell receptor [11] and results not shown). Imaging studies have provided evidence for a PLC (phospholipase C)/Ca²⁺/DAG (diacylglycerol)-dependent pathway that addresses unpalmitoylated Ras at endomembranes via the DAG-dependent recruitment of the GEF (guanine-nucleotide-exchange factor) RasGRP1 [21,22]. Phorbol ester PMA acts as a DAG analogue to engage RasGRP1 [23,24] and has been proposed to be a genuine activator of endomembrane resident N-Ras and H-Ras in COS and T-cells [21,22]. To collect biochemical evidence for or against this scenario we challenged COS-7 cells with PMA and monitored activation of wild-type compared with palmitate-null N-RasC181S. As reported previously [16,18], Ras–GTP accumulation in response to PMA was pronounced and prolonged as opposed to the more transient response elicited by EGF (Figure 1D). Remarkably, palmitate-free HA–N-RasC181S was not activated by PMA (Figures 1E and 1F). To exclude technical constraints in the detection of unpalmitoylated N-Ras–GTP, we analysed a panel of constitutively GTP-loaded N-RasG12V mutants including the palmitoylation-free version N-RasG12V/C181S (Figure 1G). N-RasG12V/C181S scored in the Ras activation assay and was undistinguishable from its palmitoylation-competent counterpart N-RasG12V. Detection of palmitoylation-free N-Ras–GTP was specific because the effector-site point mutation D38A which abrogates binding to the RBD affinity probe used to assay Ras activity [25] impaired detection of N-RasG12V/C181S/D38A. Taken together, the findings of the present study indicated that palmitate-free N-Ras was not significantly activated by EGF or PMA.
N-Ras activated in response to growth factors is palmitoylated

The conclusion above, conversely, implied that N-Ras molecules that became GTP-loaded in response to EGF or PMA should be palmitoylated. To test this assumption we metabolically labelled COS-7 cells with \(^{3}H\)palmitate and assessed the palmitate content of GTP-loaded N-Ras. As seen in the fluorography of the Ras–GTP samples in Figure 2(A), the N-Ras molecules that became GTP-loaded in response to EGF did indeed contain palmitate. The GTP-loaded palmitoylation mutant N-RasG12V/C181S did not incorporate any label, proving that the fluorography signal stemmed from post-translational palmitoylation rather than from the metabolization of the administered \(^{3}H\)palmitate. Owing to a number of unknowns the data in Figure 2(A) cannot provide conclusive information about the stoichiometry of Ras palmitoylation [17]. For example, the specific activity of palmitate in metabolically labelled cells cannot be easily determined. Moreover, it is arduous to quantify or even estimate the extent of Ras-bound \(^{3}H\)palmitate loss during electrophoresis and other sample processing steps. This notwithstanding, since the \(^{3}H\)palmitate content of N-Ras–GTP exhibited a pattern that was essentially superimposable on the Ras–GTP protein profile we concluded that the fractional palmitoylation of activated N-Ras–GTP did not greatly vary throughout the course of stimulation. Since palmitate-free N-Ras does not become activated by EGF in the first place (Figure 1B), these data are most consistent with the view that the majority of N-Ras molecules activated in response to EGF were palmitoylated. Recapitulation of the same experiment in cells treated with PMA yielded the same outcome, i.e. virtually congruent profiles of \(^{3}H\)palmitate and Ras protein in Ras–GTP pull-down assays (Figure 2B), indicating as before that the bulk of agonist-activated N-Ras was palmitoylated. Owing to low signal yield these experiments required overexpression of N-Ras so as to raise the \(^{3}H\)palmitate signal on Ras. In order to record palmitoylation of endogenous N-Ras–GTP, we used MCF-7 cells, a mammary cancer cell line that features N-Ras gene amplification, resulting in severalfold increased N-Ras protein levels [15,26,27]. Accordingly, N-Ras represents approximately 80–90% of growth-factor-activated Ras in MCF-7 cells (Figure 2C). As seen before in COS-7 cells, the palmitate content of EGF-activated endogenous N-Ras–GTP matched the time course of N-Ras–GTP formation (Figure 2C), consistent with a high and constant degree of Ras–GTP palmitoylation. This notion was supported further by the parallel assessment of palmitate content in Ras–GTP and Ras–GDP sequentially purified out of EGF-challenged MCF-7 cells (Supplementary Figure 1 at http://www.biochemj.org/bj/454/bj4540323add.htm), which showed that palmitoylation of Ras–GTP was not inferior to that of Ras–GDP.

Dominant-negative RasS17N requires palmitoylation for its inhibitory action

The findings above demonstrated that Ras palmitoylation was an important requisite for growth-factor-dependent N-Ras activation. In order to scrutinize this notion further, we studied the dominant-negative Ras mutant RasS17N. RasS17N inhibits growth-factor-induced Ras [12,28] and ERK [29,30] activation via the sequestration of GEFs, although other features of RasS17N can add to its dominant-negative action [31,32]. Since the blocking function of RasS17N therefore relies on
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Figure 3  RasS17N requires palmitoylation to exert its dominant negative effect

(A) COS-7 cells with inducible expression of Myc–H-RasS17N or the palmitoylation-deficient variant Myc–H-RasS17N/C181S/C184S were cultured for 36 h in the presence (+) or absence (−) of ATC to repress or induce dominant negative Ras expression respectively. Cells were stimulated with 10 ng/ml EGF and Ras–GTP levels and phosphorylation of ERK and Akt were determined as described in the Experimental section. Total and active Ras was detected using a pan-Ras antibody. Experiments with a second clone for each background produced the same results. End. Ras, endogenous Ras. (B) Densitometric quantification of data shown in (A). Black columns, + ATC; white columns, − ATC. Levels of active Ras (n = 4), ERK1/2 (n = 4) and Akt (n = 3) were normalized to total protein levels and are depicted as means ± S.E.M. The resting value in non-stimulated point of + ATC uninduced series was arbitrarily set to 1 in all panels.

The usurpation of Ras-GEFs, we hypothesized that if the absence of palmitoylation compromised Ras activation by GEFs it should by analogy preclude the inhibition of GEFs by RasS17N and lessen its inhibitory potency. In order to test this hypothesis we generated cell lines with inducible expression of dominant-negative H-RasS17N versions. For these experiments we used H-RasS17N since it is by far the most frequently used variant and because H-RasS17N possesses the strongest blocking potency and widest spectrum of inhibition among the three Ras isoforms [33]. As reported previously, H-RasS17N caused a pronounced (approximately 60–70%) inhibition of EGF-dependent Ras activation, which translated into markedly reduced activation of the downstream Ras-target ERK (Figure 3A, quantification shown in Figure 3B). Activation of a second Ras effector branch, the PI3K (phosphoinositide 3-kinase)/Akt pathway, was not affected, suggesting that EGF activated PI3K via Ras-independent mechanisms in COS cells [34]. By contrast, EGF-driven activation of Ras and ERK was not blocked, nor was it even partially affected by palmitoylation-deficient HA–RasS17N/C181S/C184S, indicating that H-RasS17N required
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N-Ras activation proceeds at the PM

Palmitoylation dictates the subcellular distribution of N-Ras, with palmitoylated N-Ras residing at the PM, whereas depalmitoylated N-Ras is largely associated with endomembranes [3,20]. Thus another implication of the findings above was that N-Ras activation occurred at the PM and less so at endomembranes. This notion, in principle, contradicts previous studies that documented the presence of N-Ras–GTP or H-Ras–GTP at the Golgi of agonist-stimulated MDCK (Madin–Darby canine kidney) [4], COS [21,35] or T-cells [22]. In the previous studies the ability to specifically visualize N-Ras–GTP was contingent on the overexpression of N-Ras, raising the concern that Ras overexpression might disturb the regulation of RasGDP/GTP levels by GEF/GAP (GTPase-activating protein) systems and/or Ras subcellular distribution [36,37]. In line with that possibility, we observed previously that overexpression of H-Ras along with RBD-based affinity probes causes a marked growth-factor-independent accumulation of Ras–GTP at the Golgi apparatus of COS cells [12], suggesting that endomembrane Ras may possibly be subject to more loose control by GAPS than Ras located at the PM. In the light of these considerations and in order to avoid Ras overexpression while still being able to selectively visualize N-Ras–GTP, we imaged Ras–GTP formation in MCF-7 cells. As mentioned previously, N-Ras constitutes up to 90 % of growth-factor-activated Ras in MCF-7 cells (Figure 2C), implying that any Ras–GTP formation detected in imaging experiments was largely attributable to N-Ras. Live-cell Ras activation was visualized using E3-R3(A/D), a trivalent high-avidity/high-fluorescence reporter probe for Ras–GTP composed of three EGFP and three attenuated RBD modules [11,12] (Supplementary Figure 2 at http://www.biochemj.org/bj/454/bj4540323add.htm). MCF-7 cells expressing E3-R3(A/D) were labelled with the vital Golgi marker BODIPY® TR C5 Ceramide and challenged with 50 ng/ml EGF and imaged confocally. EGF-induced activation of the mitogenic ERK1/2 MAPK pathway was blunted in Rasless MEFs (Figure 5A). The P3K/Akt pathway was also affected, whereas activation of Raf, a GTPase activated downstream of a third group of Ras effectors comprising three Raf-GEFs, was not altered, indicating that ERK activation independently of Ras in MEF cells. As expected, introduction of wild-type N-Ras via lentiviral transduction restored both EGF-dependent ERK activation (Figure 5B) and serum-driven proliferation (Figure 5C), illustrating that N-Ras functioned as a mediator in mitogenic pathways. N-Ras-driven proliferation was not as robust as the one elicited by K-Ras in this system [39]. In contrast, the palmitoylation-deficient variant N-RasC181S failed to rescue or even partially restore EGF-dependent ERK activation and serum-driven proliferation (Figures 5B and 5C), confirming that the lack of palmitoylation rendered N-Ras incompetent for mitogenic signalling.
DISCUSSION

The findings in the present study document a critical role of palmitoylation for the biological function of N-Ras as a mediator of growth-factor-elicited signals. In particular, our data argue against the existence of a signalling pathway genuinely devoted to the localized activation of unpalmitoylated N-Ras at endomembranes, at least in the cell types investigated in the present study, since the palmitoylation-deficient N-Ras variants used throughout our experiments reside mostly in that compartment. One reported mechanism for endomembrane-activation of N-Ras and H-Ras involves the activation of the GEF RasGRP1 in situ at the Golgi via a PLC/Ca\(^{2+}\)/DAG-dependent pathway [21]. Since RasGRP1 is a neuronal and leukocyte-specific GEF [40,41], this pathway is unlikely to operate in cell types such as COS or MDCK, both of which reportedly do not express meaningful amounts of RasGRP1 [18,42]. The absence of RasGRP1 could thus suffice to explain the lack of activation of palmitoylation-deficient N-Ras in COS, MEF or MCF-7 cells documented in the present paper although, paradoxically, the existence of a PLC/DAG/RasGRP1 pathway of endomembrane Ras activation was invoked on the basis of findings obtained from COS cells [21,35]. Moreover, RasGRP1 deficiency cannot account for the previously reported lack of N-RasC181S activation in Jurkat T-cells [11], since those cells express high levels of RasGRP1.

A second mechanism for endomembrane Ras activation involves the relocation of growth-factor-activated PM-resident
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Ras–GTP to endomembranes following its depalmitoylation at the PM [3,43,44]. Although an increasing body of data has firmly established the fundamental validity of the acylation–deacylation cycle model for endomembrane relocation of Ras, it remains difficult to assess its dimension and importance as a mode of endomembrane Ras activation. One caveat relates to the fact that all experimental evidence stems from overexpression studies in which normal Ras activity control can potentially be distorted. For example, the lifetime of Ras–GTP complexes could be affected by the degree of Ras overexpression and/or by the RBD probes used for Ras–GTP visualization (which impede GAP action on Ras [45]), enabling depalmitoylated N-Ras–GTP to endure the journey to endomembranes. This effect could, e.g. be responsible for the previously observed stimulus-independent presence of Ras–GTP at endomembranes of RBD-expressing cells [12,46]. With regard to the data in the present study, the biochemical analysis of N-RasC181S activation cannot provide evidence for or against this mode of endomembrane Ras activation since N-RasC181S is not a substrate for the acylation–deacylation cycle. N-RasC181S can thus only report on Ras activation processes that proceed in situ at endomembranes and/or those targeting specifically unpalmitoylated N-Ras. On the other hand, the imaging experiments shown in Figure 4 do not support the model, since they document an absence of N-Ras–GTP accumulation at endomembranes.

Even though a number of factors (such as the increase in cytosolic mean fluorescence as the E3-R3 probes fall off the PM at later time points of stimulation) can potentially hamper the detection of endomembrane located Ras–GTP, the fact that endogenous N-Ras–GTP formation was readily visible at the PM of the same cells suggests that endomembrane Ras–GTP levels formed in response to EGF must, at best, be negligibly low. Thus although a negative result is not proof of absence, together with previous studies that similarly did not detect endogenous Ras–GTP formation at endomembranes [11,12], the present data argue for the PM as the relevant platform of N-Ras activation and signal transmission in growth factor signalling. However, our findings do not exclude a function of endomembrane, palmitate-free Ras–GTP in the transmission of physiological or pathophysiological signals elicited by cues other than the mitogens tested in the present study. Notably, in some overexpression experiments such as the one shown in Figure 1(B), but not in others, a minor fraction of unpalmitoylated N-Ras apparently became GTP-loaded in response to EGF. As speculated before by others [47], we attribute this minor pool of GTP-loaded palmitate-free N-RasC181S–GTP to a small population of PM-resident N-RasC181S because the very same N-RasC181S protein never loaded in response to EGF. As speculated before by others [45], increased PM off-rates and others do not suffice to frustrate Ras–GTP visualization (which impede GAP action on Ras [45]), enabling depalmitoylated palmitate-free N-RasC181S–GTP to a small population of PM-resident N-RasC181S–GTP to a small population of PM-resident N-RasC181S because the very same N-RasC181S protein never

The simplest interpretation is that unpalmitoylated N-Ras, even if partly associated with the PM, is neither a target nor a mediator in growth factor signalling, a notion reinforced by previous findings documenting an absolute requirement of N-Ras palmitoylation for onset and progression of haematologic malignancies driven by oncogenic N-Ras [56,57]. However, as mentioned before, the findings do not rule out that palmitate-free Ras could fulfil physiological functions in other cellular backgrounds, perhaps in dependency of cell type-specific variables such as the GEF/GAP ratio at the PM compared with endomembranes or the relative flux through the acylation–deacylation cycle.

In summary, our data illustrate that activation of N-Ras proceeds at the PM and are most consistent with a scenario in which depalmitoylation of N-Ras serves as a mechanism for the down-regulation of Ras signalling rather than for the relocation of Ras signalling from PMs to endomembranes.

AUTHOR CONTRIBUTION

Shu-Ping Song, Anne Hennig, Katja Schubert, Robby Markwart, Philipp Schmidt and Ian Prior performed the experiments; Ian Prior conceived the study; Ian Prior, Frank Bohmer and Ignacio Rubio designed the experiments; and Ignacio Rubio wrote the paper.

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SUPPLEMENTARY ONLINE DATA
Ras palmitoylation is necessary for N-Ras activation and signal propagation in growth factor signalling

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Figure S1 Growth-factor-activated GTP-loaded Ras is preferentially palmitoylated compared with Ras-GDP

MCF-7 cells grown in 6-cm-diameter dishes were grown to sub-confluency and deprived of serum overnight before metabolic labelling with [3H]palmitate (3 mCi/dish) as described in the main text. Cells were stimulated with EGF for various periods of time and Ras GTP was pulled out of cell extracts with immobilized GST–RBD as described in the main text. The cell extracts depleted of Ras–GTP were then subjected to a Ras-IP (immunoprecipitation) with the Y13-259 monoclonal rat antibody [1] to collect Ras–GDP left in the lysate. Ras–GTP and Ras–GDP samples were split in a ratio of 1:2 (33%:66% as indicated) loaded on to the same SDS/PAGE gels and subjected to Western blotting with anti-N-Ras antibody or fluorography. Bands were quantified and plotted as the ratio of Ras-bound radioactivity to protein. The palmitate/protein ratio for Ras–GTP in resting cells was arbitrarily set to 1. A second experiment showed essentially the same result.

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E3-R3(A/D) was electroporated into MCF7 cells in combination with plasmids coding for various mCherry-tagged N-Ras variants, including palmitoylation-less, endomembrane-resident N-RasC181S mutants. The cells were imaged confocally 24 h later. E3-R3(A/D) decorates GTP-loaded N-Ras both at the PM and endomembrane. Ras-GTP visualization is specific because co-localization was abrogated by the effector-site mutation D38A, which is known to compromise the Ras-GTP–RBD interaction [2]. Note that expression of oncogenic GTP-loaded N-RasG12V causes extensive membrane ruffling, if located on the PM, and Golgi fragmentation if present on endomembranes. Both effects are reversed by the effector-site mutation D38A. All scale bars are 10 μm.

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