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Ras isoform abundance and signalling in human cancer cell lines

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Abstract

The ubiquitously expressed major Ras isoforms: H-, K- and N-Ras, are highly conserved, yet exhibit different biological outputs. We have compared the relative efficiencies with which EGF or HGF activate Ras isoforms and the requirement for specific isoforms in the activation of downstream pathways. We find that the relative coupling efficiencies to each Ras isoform is conserved between stimuli. Furthermore, in both cases, inhibition of receptor endocytosis led to reduced N-Ras and H-Ras activation, but K-Ras was unaffected. Acute knockdown of each isoform with siRNA allows endogenous Ras isoform function and abundance to be probed. This revealed that there is significant variation in the contribution of individual isoforms to total Ras across a panel of cancer cell lines although typically K≥N>>H. Intriguingly, cancer cell lines where a significant fraction of endogenous Ras is oncogenically mutated showed attenuated activation of canonical Ras effector pathways. We profiled the contribution of each Ras isoform to the total Ras pool allowing interpretation of the effect of isoform specific knock-down on signalling outcomes. In contrast to previous studies indicating preferential coupling of isoforms to Raf and PtdIns-3-kinase pathways, we find that endogenous Ras isoforms show no specific coupling to these major Ras pathways.

Keywords

Ras; siRNA; Akt; HGF; EGF

INTRODUCTION

The p21 Ras family of monomeric GTPases function as molecular switches relaying signals from cell surface receptors to intracellular kinase cascades regulating cell proliferation, differentiation, apoptosis and senescence (Hancock, 2003). The three ubiquitously expressed Ras isoforms: H-Ras, K-Ras 4B (hereafter referred to as K-Ras) and N-Ras, share almost complete sequence homology between amino acids 1-165. Studies indicating identical *in vitro* effector activation profiles (Wittinghofer & Herrmann, 1995) implied that the isoforms were functionally redundant. However, tumour profiling, knock-out mice, localisation and over-expression studies have provided evidence for Ras isoform-specific signalling (reviewed in (Hancock, 2003).

Mechanisms that regulate isoform-specific signalling include differential compartmentalisation within cell surface microdomains and intracellular compartments (Chiu et al., 2002; Prior et al., 2003; Roy et al., 1999) and coupling to activators and effectors specific for individual, or a subset of Ras isoforms (Mitin et al., 2004; Villalonga et

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al., 2001; Vos et al., 2003). A simple mechanism is through regulation of the cellular expression profiles but scant quantitative data exist for relative levels of cellular Ras protein expression.

There are potential problems associated with each approach hitherto adopted to investigate Ras isoform specific signalling. Cells derived from knock-out animals may have developed compensatory mechanisms which could over-emphasise redundancy. In experiments when constitutively active Ras mutants are ectopically expressed, Ras activation is both uncoupled from specific receptor stimulation and the amount of expressed protein typically exceeds endogenous levels. Importantly, differences in overexpressed versus endogenous Ras signalling profiles have been observed. Oncogenic Ras over-expression in normal human fibroblasts results in replicative senescence whereas mouse embryonic fibroblasts derived from mice engineered to express endogenous levels of oncogenic Ras are immortalised but not senescent (Serrano et al., 1997; Tuveson et al., 2004).

We have now examined Ras isoform-specific signalling in HeLa cells using acute knock-down with siRNA oligos directed against specific Ras isoforms. This approach allows us to monitor endogenous signalling without the complicating factors outlined above. We can estimate the relative amounts of each isoform by relating the loss of a specific isoform to the reduction in total Ras. By also measuring the efficiency of activation of each Ras isoform we can obtain the contribution of each isoform to the total Ras activation profile in response to growth factors. Thus whilst differential Ras isoform-receptor coupling is observed, our results indicate no requirement for specific endogenous Ras isoforms to activate the Raf and PtdIns-3-kinase (PI3K) pathways.

RESULTS

Activation of individual Ras isoforms in response to HGF and EGF

Ras proteins exhibit different compartmentalised distributions within the plasma membrane, and this may regulate access to different pools of activators and effectors (Hancock, 2003). The growth factors HGF and EGF both stimulate tyrosine kinase receptors Met and EGFR respectively. In distinction to EGFR, which utilises seven phosphotyrosine residues to recruit substrates and adaptors, Met receptor has a minimal substrate-docking site incorporating only two phosphotyrosine residues and relies on the Gab1 adaptor protein to diversify its signalling outputs. Furthermore, stimulation of Met leads to a wider variety of cellular effects (Birchmeier et al., 2003). We wondered if a particular Ras isoform was preferentially coupled to either receptor, which might then reflect differential micro-localisation at the plasma membrane. HeLa cells were stimulated with saturating concentrations of either EGF or HGF for use in pull-down assays of activated Ras, using a GST fusion of a high affinity Raf-1 Ras binding domain (RBD K85A) (Fridman et al., 2000). EGF stimulation results in a greater activation of all the Ras pool compared to HGF, but the rank order of Ras isoform activation remained the same regardless of ligand. The ratio of the specific “pull down” intensity to “lysate” intensity for each isoform provides a relative measure of the efficiency of activation. Quantitative analysis of Figure 1A suggests that N-Ras is activated to a slightly higher extent than K-Ras, with H-Ras being activated to the least degree (Fig. 1A). The same rank order was preserved at sub-saturating ligand concentrations (data not shown). After 30 minutes, activation of H-Ras is markedly attenuated after both EGF and HGF stimulation, whereas this effect is modest for the other two isoforms (Figure 1B).

Inhibition of receptor endocytosis reduces H- N-, but not K-Ras activation

Activation of both Met and EGFR leads to rapid internalisation of receptors to endosomes (Haigler et al., 1978; Hammond et al., 2001). In endocytically compromised HeLa cells, EGF- but not HGF-dependent MAP kinase (MAPK) activation is markedly impaired (Hammond et al., 2003; Vieria et al., 1996). We asked if activation of any endogenous Ras isoform may be restricted to this compartment. We employed a stably transfected HeLa cell line which when cultured in the absence of tetracycline expresses a dominant-negative form of dynamin (K44A), a protein essential for endocytosis (van der Bliek et al., 1993). Immunofluorescence indicated >90% of the cells in culture expressed HA-tagged K44A dynamin upon tetracycline withdrawal, and were unable to internalise ligand-activated Met or EGFR (data not shown, (Hammond et al., 2001)). Expression of K44A dynamin resulted in a marked decrease in the amount of activated H- and N-Ras pulled-down with GST-RBD following 5 minutes stimulation with either EGF or HGF. However, K-Ras activation is independent of endocytosis (Figure 1C).

Contribution of Ras isoforms to the total Ras pool

We investigated the relative abundance of each isoform, so that its actual contribution to the activated Ras pool could be determined. Isoform-specific siRNA duplexes to H-, K- or N-Ras were used to individually knock-down Ras isoforms by RNA interference resulting in ≥90% knock-down of each Ras isoform (Figure 2A). In order to determine the level of expression of each isoform, pan-Ras antibodies were used to measure the total cellular Ras remaining following each isoform-specific knock-down, relative to that seen in both non-targeting, “scrambled” oligonucleotide-treated control cells, or vehicle-only control cells (Figure 2A). Of several tested (including sc-32: Santa Cruz and #3965: Cell Signalling; not shown), only monoclonal Y13-259 was a *bona fide* pan-Ras antibody recognising each isoform with equal efficiency (Figure 2B). Probing with Y13-259 allowed us to estimate the contribution of each isoform (H-, K- or N-Ras) to the total Ras pool from the reduction in intensity of Ras bands on Western blots of cell lysates from knock-down cells compared with controls.

Our data indicate that in HeLa cells there are approximately equal amounts of K- and N-Ras (40 - 45% of total), but much less H-Ras (<10% of total) (Figure 2A). K- and N-Ras run as distinct bands within a doublet on high-resolution gradient gels, with K-Ras almost exclusively the higher molecular weight upper band of the doublet, and N-Ras accounting for the vast majority of the lower molecular weight immuno-reactive band. The very small H-Ras component makes up the remainder of the lower band immuno-reactivity (Figure 2A). We extended these studies to examine Ras isoform abundance in a panel of tumour derived cell lines (Figure 3). Significantly, in all cell lines with the exception of HEK293T and HepG2, K-Ras represents the most abundant isoform whereas H-Ras is typically expressed at low levels except in lung derived tumour cell lines. Analysis of the variance of isoform abundance reveals that across cell lines individual isoforms exhibit a significant degree of variation in expression. Specifically, H-Ras (>50-fold or 7-fold when lymphoid cells not included), K-Ras (4.5-fold), N-Ras (6-fold); revealing significant tuning of individual isoforms, depending on the cellular context. Finally, there appears to be no significant correlation between Ras isoform abundance and preferential mutation of isoforms associated with particular cancers for example K-Ras and pancreatic cells (Panc-1, SUIT-2) or N-Ras and leukaemia cells (Bos, 1989). Indeed in B cell leukaemia derived samples, N-Ras abundance is at its lowest across the panel of cells (15-20%) whereas K-Ras represents approximately 80% of total Ras.

By combining our estimates for the representation of each Ras isoform with the efficiency of activation, we can dissect the Ras activation profile into component parts. Thus, although H-

Ras is efficiently activated in HeLa cells by both HGF and EGF (Figure 1), its contribution to the total Ras signal will be small. Estimates of isoform abundance are especially informative when analysing the pattern of effector activation within the cancer cell lines. In these samples we observe constitutive activation of Akt and MAPK that is independent of the predicted activation status of Ras or other regulatory proteins (Figure 4A). For example, DU145 cells possessing normal Ras, PI3K, PTEN and Raf (Sanger), display similar or even enhanced activation of Akt and MAPK in comparison with cell lines harbouring oncogenically mutated Ras. Despite the suppression of signalling, cell lines possessing mutated oncogenic Ras isoforms (HepG2: N-Ras^{Q61L}; A549: K-Ras^{G12S}) retain the ability to display an acute effector activation profile comparable to that seen for control cells (Figure 4B). Therefore, these cancer cell lines have largely uncoupled the oncogenic Ras proteins from downstream effector pathways without perturbing the ability of the remaining Ras proteins from promoting signalling in response to growth factor stimulation.

Isoform coupling to effector pathways

Quantitative determination of the activation of Ras isoforms has enabled us to ask if the knock-down of specific isoforms influences downstream signalling events proportionate to their contributions to the activated Ras pool. Any deviations are indicative of isoform specificity in that signalling pathway. Activation of specific effectors could be coupled to specific Ras isoforms through coincident micro-localisation. Downstream signalling events are exemplified by activation of MAPK and Akt/PKB. In control HeLa cells, acute stimulation with EGF or HGF leads to a transient activation of both MAPK and Akt (Figures 5 and 6A), which peaks around 5 minutes and subsequently attenuates through dephosphorylation. All experiments shown used 100ng/ml of growth factor which represents a saturating dose however, identical results were obtained with sub-saturating 10ng/ml of HGF (data not shown).

Examination of MAPK phosphorylation indicates functional redundancy amongst isoforms for both EGF and HGF stimulation (Figure 5). Knocking down all three Ras isoforms abrogated MAPK activation (data not shown) however, knocking down any one individual Ras isoform has no marked effect on the extent and kinetics of MAPK activation. This suggests that isoform specific differences are not due to differential signalling through the MAPK cascade.

Similarly, we observed minimal Ras isoform specific effects in the regulation of PKB/Akt phosphorylation. K-Ras displays the strongest correlation between the degree of knockdown and the level of inhibition of Akt phosphorylation across 5 different siRNA oligos (Figure 6). In contrast, for both H- and N-Ras we see variable levels of phospho-Akt that does not correlate with the extent of Ras isoform knockdown. For H-Ras, oligos H2 and H3 have produced almost complete knockdown but phospho-Akt remains at control levels indicating that the decreases seen for the other H-Ras oligos are likely non-specific RNAi effects. This is confirmed using re-complementation experiments with RNAi-resistant Ras isoforms (Figure 6B); even with significant over-expression of H-Ras (>50% transfection efficiency, data not shown), phospho-Akt levels do not return to control levels. For N-Ras, knockdown generally produces a reduction in phospho-Akt to a similar extent to that seen for K-Ras. However the extreme examples seen with oligos N1 and N5 are also likely to be non-specific since N-Ras add back fails to restore phospho-Akt to control levels (Figure 6B). Therefore for future knockdown studies of H- and N-Ras, oligos H3, H4, H6, N2, N3 and N4 should be used to avoid non-specific effects on the PI3K-Akt pathway. In summary, whilst we observe a reduction of phospho-Akt levels proportional to Ras isoform abundance, there appears to be no preferential coupling of either MAPK or PI3K signalling to any endogenously expressed Ras isoform.

DISCUSSION

In recent years it has become apparent that Ras isoforms do not show homogeneous distributions on the plasma membrane (Hancock, 2003). For example H-Ras is more concentrated in cholesterol rich microdomains (Prior et al., 2003). This leads to the notion that activation of different isoforms may be more or less tightly coupled to particular receptors, depending on the degree to which they co-localise in specific categories of plasma membrane domain. We have analysed the isoform specificity of endogenous Ras activation in HeLa cells downstream of HGF and EGF stimulation. Both of these growth factors bind to tyrosine kinase receptors, which undergo autophosphorylation upon stimulation and recruit the Ras exchange factor Sos through the adapter protein Grb2, which binds to phosphotyrosine residues in the activated receptors. Both stimuli result in activation of well-characterised Ras-dependent pathways leading to MAP kinase and PKB/Akt activation respectively. However, they have divergent effects on cell phenotypes with HGF being a significantly more versatile and powerful motogen and morphogen (Birchmeier et al., 2003). In endocytically compromised HeLa cells, EGF-dependent MAPK activation is markedly impaired (Vieria et al., 1996), whereas HGF-dependent activation is largely unaffected (Hammond et al., 2003).

Our data revealed a rank order of activation of Ras isoforms that is conserved between EGF and HGF stimulation, suggesting that their signalling outcomes do not diverge as a consequence of differing plasma membrane distributions of receptors. In accordance with previous studies of Ras effector activation using constitutively activated Ras isoforms uncoupled from receptor stimulation (Roy et al., 2002), we have now shown that EGF or HGF-dependent activation of H-, N- but not K-Ras is also reduced when endocytosis is blocked by dominant-negative dynamin. Consequently, differences in Ras activation profiles cannot underpin the different sensitivities of EGF- and HGF-dependent MAPK activation to blockade of endocytosis.

To analyse endogenous Ras isoform signalling we used RNAi technology to acutely knockdown specific isoforms. The development of siRNA strategies for mammalian cells has led to the ability to knock-down cellular protein expression in a highly specific and effective manner (Elbashir et al., 2001), such that previous strategies using anti-sense oligonucleotides have largely been superceded (Chen et al., 1996; Sharpe et al., 2000). Using this approach we have been able to specifically knock-down Ras isoforms by >90% in each case. This has allowed us to estimate the contribution of each isoform to the total Ras pool from the reduction in intensity of pan-Ras antibody bands in Western blots of cell lysates. This provides an alternative strategy to that used in previous studies, isoform specific antibodies have been calibrated against Ras protein standards (Sharpe et al., 2000; Wolfman & Wolfman, 2000). Our data indicate roughly equal amounts of K-Ras and N-Ras, which run as distinct bands on high-resolution gradient gels with little H-Ras present in HeLa cells. Low levels of H-Ras have also been reported in human renal fibroblasts (Sharpe et al., 2000) and inferred in a number of other cell types by a failure to detect H-Ras with isoform specific antibodies (Giehl et al., 2000). Our data now reveal that H-Ras is a relatively minor isoform in many cell lines. Intriguingly, the two most abundant isoforms K(B)-Ras and N-Ras represent the extreme ends of the spectrum of Ras localisation. K(B)-Ras is almost exclusively cell surface associated whereas N-Ras displays a prominent ER/Golgi pool in many cell types.

Understanding the relative abundance of Ras isoforms helps inform our interpretations of their role in signalling and phenotypic outputs. For example, our data examining effector activation in cancer cell lines expressing endogenous oncogenic Ras isoforms revealed that activation of downstream signalling was not always evident. A lack of Raf-MAPK

activation has been observed previously in pancreatic cell lines, leukaemia cells and mouse embryonic fibroblasts from a mouse model expressing oncogenic K-Ras (Giehl et al., 2000; Iida et al., 1999; Seufferlein et al., 1999; Tuveson et al., 2004; Yip-Schneider et al., 1999). Our data extends these observations, revealing that lung and liver derived cancer cell lines exhibit similar attenuation and that the PI3K-Akt pathway is also uncoupled from oncogenic Ras signalling. Our analysis of isoform abundance indicated that this was not due to the oncogenic isoform being expressed at low levels. For example, in Panc-1 or SUIT-2 pancreatic cell lines, K-Ras represents 60% of the total Ras. However, despite this translating into 30% of total Ras being constitutively active, effector activation resembles that seen in non-Ras mutated cells such as DU145 and MCF7. This indicates that the suppression of signalling is more likely due to specific uncoupling of the mutated Ras isoform from the effector pathways. A specific mechanism for this was described in previous work that characterised up-regulation of MAP kinase phosphatase-2 activity in pancreatic cells expressing oncogenic K-Ras (Yip-Schneider et al., 2001). Whilst this may in part explain the lack of phospho-ERK that we observed across a range of cancer cell lines harbouring oncogenic Ras isoforms, we also observed attenuation of Akt activation. Clearly, multiple regulatory mechanisms exist that our data indicate can be overcome by acute stimulation with serum. This may mean that a threshold has been overcome of inhibitory regulators such as phosphatases responsible for dampening oncogenic Ras signalling to undetectable levels. Alternatively, acute serum stimulation may induce secondary signals that potentiate signalling through the Ras effector pathways as has been described with integrins and HGF signalling (Trusolino et al., 2001).

By combining our estimates for the representation of each Ras isoform with the efficiency of activation, we can dissect the endogenous Ras signalling profile into component parts for the first time. Quantitative determination of the activation of Ras isoforms has enabled us to ask if the knock-down of specific isoforms influences downstream signalling events proportionate to their contributions to the activated Ras pool. Any deviations are indicative of isoform specificity in that signalling pathway. We found no preferential coupling of endogenous Ras isoform signalling to the canonical Ras effector pathways: Raf-MAPK and PtdIns-3-kinase-Akt. This is in contrast to previous studies that characterised preferential coupling of H-Ras with PtdIns-3-kinase and K-Ras with Raf and Rac activation (Walsh & Bar-Sagi, 2001; Yan et al., 1998). These studies relied however on transient over-expression of constitutively active isoforms, which has been shown to generate different signalling outcomes relative to endogenous oncogenic Ras signalling (Serrano et al., 1997; Tuveson et al., 2004). Finally, we have restricted our analysis to two effector pathways; other important effectors include RalA and RalB that are differentially required for Ras-dependent pancreatic cell transformation and metastasis (Lim et al., 2006). An important next step will be to examine coupling of endogenous isoforms with the full spectrum of Ras effectors and their roles in specific phenotypic outputs.

In summary, we have characterised differential Ras isoform coupling to receptors and dependence on subcellular trafficking for efficient signaling. We have shown that RNAi provides a powerful tool for analysis of endogenous Ras abundance and isoform specific signalling events, when combined with quantitative estimates of the mole fraction that each isoform contributes to the activated Ras pool. The utility of these tools for generating signatures of endogenous Ras isoform signalling mean that they will be invaluable for the essential next phase of Ras research.

MATERIALS AND METHODS

Cell culture and plasmids

All cell lines, unless otherwise stated, were obtained from the CRUK cell bank. Primary human lymphoid cancer cell lysates were provided by Joe Slupsky, University of Liverpool. Normal and HA-dynamin K44A mutant HeLa cells (a generous gift of S. Schmid) were cultured as described previously (Hammond et al., 2003). Withdrawal of tetracycline 48 h before the experiment induced K44A dynamin expression; monitored by Western blotting and immunofluorescence. Expression vectors for GFP-tagged activated G12V Ras isoforms have been described previously (Prior et al., 2001). GST-RBD K85A was a generous gift of Tony Burgess, Ludwig Inst., Melbourne (Fridman et al., 2000). RNAi resistant H-Ras was made by PCR from human H-Ras (Prior et al., 2001) with the following sense and cognate antisense primers: GGGAGACGTGCCTTCTAGACATCCTGGATACCG. HA-tagged mouse N-Ras, resistant to oligos N1 and N5, was subcloned from IMAGE clone 6475312 (MRC geneservice).

Antibodies and other reagents

Ras isoform specific antibodies: polyclonal anti-H-Ras (C20) and monoclonal anti-N-Ras (F155; both Santa Cruz Biotechnology), monoclonal anti-c-K-Ras (clone 234-4.2; Sigma). Pan-Ras antibodies: monoclonal anti-v-H-Ras (clone Y13-259; Sigma) or monoclonal anti-Pan-Ras (clone F132-62; Oncogene). Monoclonal anti-phospho-p44/42 MAP kinase (Thr202/Tyr204) (E10), polyclonal anti-p44/42 MAP kinase, monoclonal anti-phospho-Akt (Ser473) (587F11) and anti-Akt were all from Cell Signalling Technology. Monoclonal anti-GFP antibody (Roche), monoclonal anti-tubulin (Sigma). Recombinant HGF was a gift from Genentech, USA and EGF was obtained from John Smith, Liverpool University.

Depletion of cellular Ras isoforms by RNA interference

The following duplexes were purchased from Dharmacon, USA: “scrambled” non-targeting siCONTROL oligonucleotides 1 and 2 (D-001210-01 and D-001210-02), H1: CGUGCCUGUUGGACAUCCU, H2: GGAAGCAGGUGGUCAUUGAUU, H3: GAACCCUCCUGAUGAGAGUUU, H4: AGACGUGCCUGUUGGACAUUU, H5: GGAAGCAGGUGGUCAUUGAUU, H6: GAGGAUGCCUUCUACACGUUU, K1: AGAGUGCCUUGACGAUACA, K2: GACAAAGUGUGUAAUUAUGUU, K3: GGAGGGCUUUCUUUGUGUAUU, K4: UCAAAGACAAAGUGUGUAAUU, K5: GAAGUUAUGGAAUUCCUUUUU, K6: GAGAUAACACGAUGCGUAUUU, N1: CUCGGAUGAUGUACCUAUG, N2: GAGCAGAUUAAGCGAGUAAUU, N3: GAAAUACGCCAGUACCGAAUU, N4: GUGGUGAUGUAACAAGAUAUU, N5: GCACUGACAAUCCAGCUAAUU.

HeLa cells were transfected with 25 nmoles (per 3.8×10^5 cells) of each siRNA duplex using oligofectamine (Invitrogen). Two days post-transfection, cells were re-transfected with siRNA; 72 h post-transfection, the cells were re-seeded into the required assay dishes and allowed to re-attach over night. Ras knock-down was confirmed by Western blotting lysates resolved on NuPAGE 4-12% Bis-Tris gels (Invitrogen). All RNAi experiments were repeated at least three times with each duplex, identical results were observed for each pair of oligonucleotides. Where indicated, RNAi-resistant Ras isoforms were transfected 24 hours before the second siRNA transfection. Ras isoform abundance was measured by quantitative analysis of Western blots using UVIchemi (UVItec, Cambridge) and Odyssey (LI-COR Biosciences) digital imaging systems. For cell line analysis of Ras isoform abundance, proportional changes in labelling were referenced to the HeLa cell values of 10:45:45 for H-:K-:N-Ras and then calculated as a % of the combined total of Ras isoform labelling. Cell line total Ras abundance was determined from pan-Ras (Y13-259) blots;

quantitation was subsequently normalised to total protein per lane (μg; specific abundance) or total protein per cell (μg / 1×10^6 cells; total abundance) and then all data referenced to HeLa cell Ras abundance.

Ras activation assay

Sub-confluent cells were serum-starved overnight before being stimulated by growth factors. The cells were then rapidly washed with ice-cold PBS and lysed on ice in 20 mM Tris-HCl, pH 7.5, 150mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, 1:250 protease-inhibitor cocktail (Sigma). 100μg of GST-RBD K85A fusion protein linked to Glutathione Sepharose(4B) was used to affinity purify activated Ras from 200μg of each lysate as described (Fridman et al., 2000). Pull downs were immuno-blotted for Ras isoforms.

Signalling assays

Control and Ras isoform-depleted cells were stimulated with the indicated amounts of growth factors, washed once with ice-cold PBS and lysed for 5-10 min on ice in a lysis buffer containing 0.5% NP-40, 25mM Tris pH 7.5, 100mM NaCl, 50mM NaF, supplemented with mammalian protease inhibitor cocktail and mammalian phosphatase inhibitor cocktail II (Sigma). MAPK and Akt activation was assayed by immunoblotting lysates with phospho-specific antibodies.

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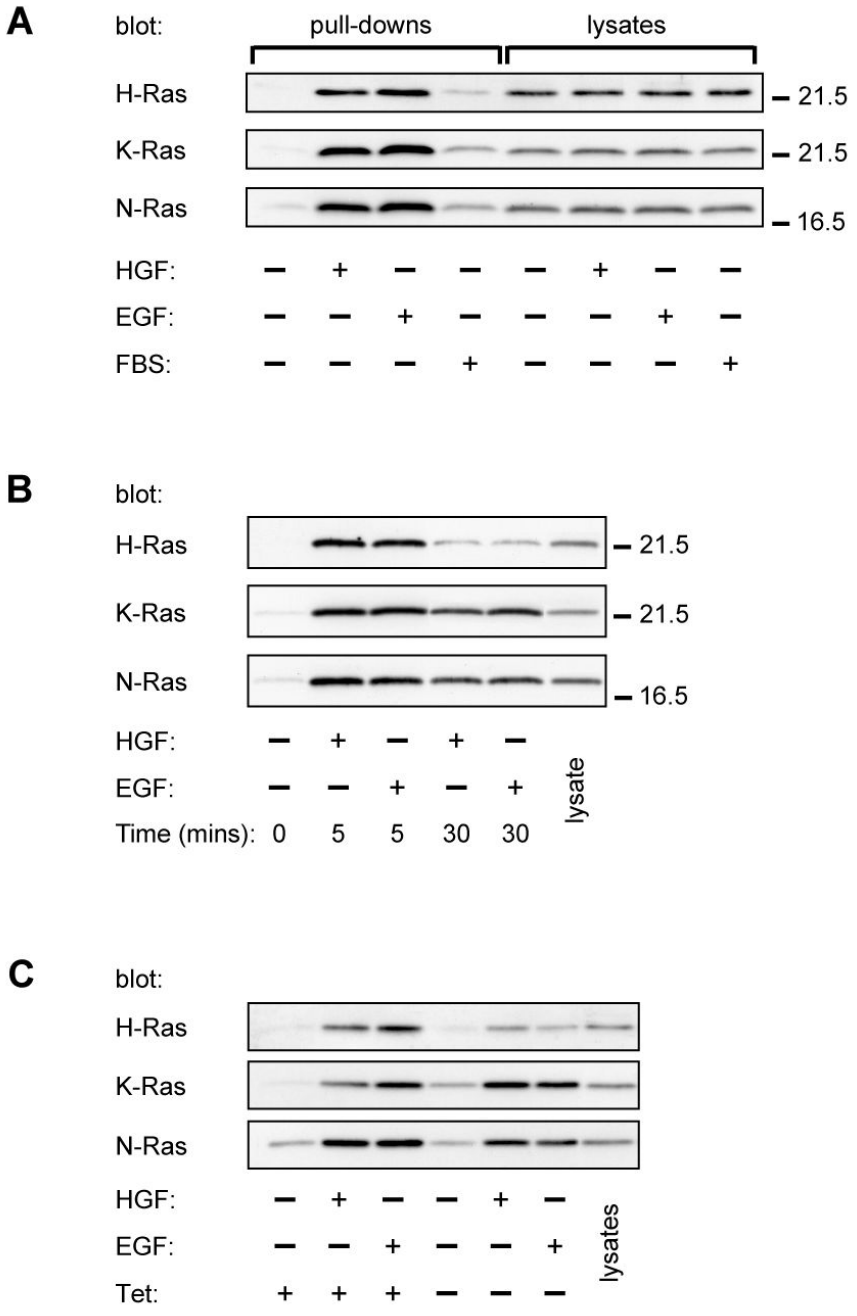

FIG. 1. Growth factor induced Ras isoform activation in normal and endocytosis-defective (K44A) HeLa cells

Serum-starved HeLa cells were stimulated with either 100ng/ml HGF or EGF for 5 min (A) or 5 and 30 min (B). Activated Ras was pulled-down using GST-RBD (K85A), 30μg of each lysate was immunoblotted with isoform-specific Ras antibodies to normalise for Ras content (B). (C) Equivalent Ras activation assays (5 minute stimulation) were performed in serum-starved K44A dynamin-expressing mutant HeLa cells. Only K-Ras activation is insensitive to inhibition of endocytosis (Tet-).

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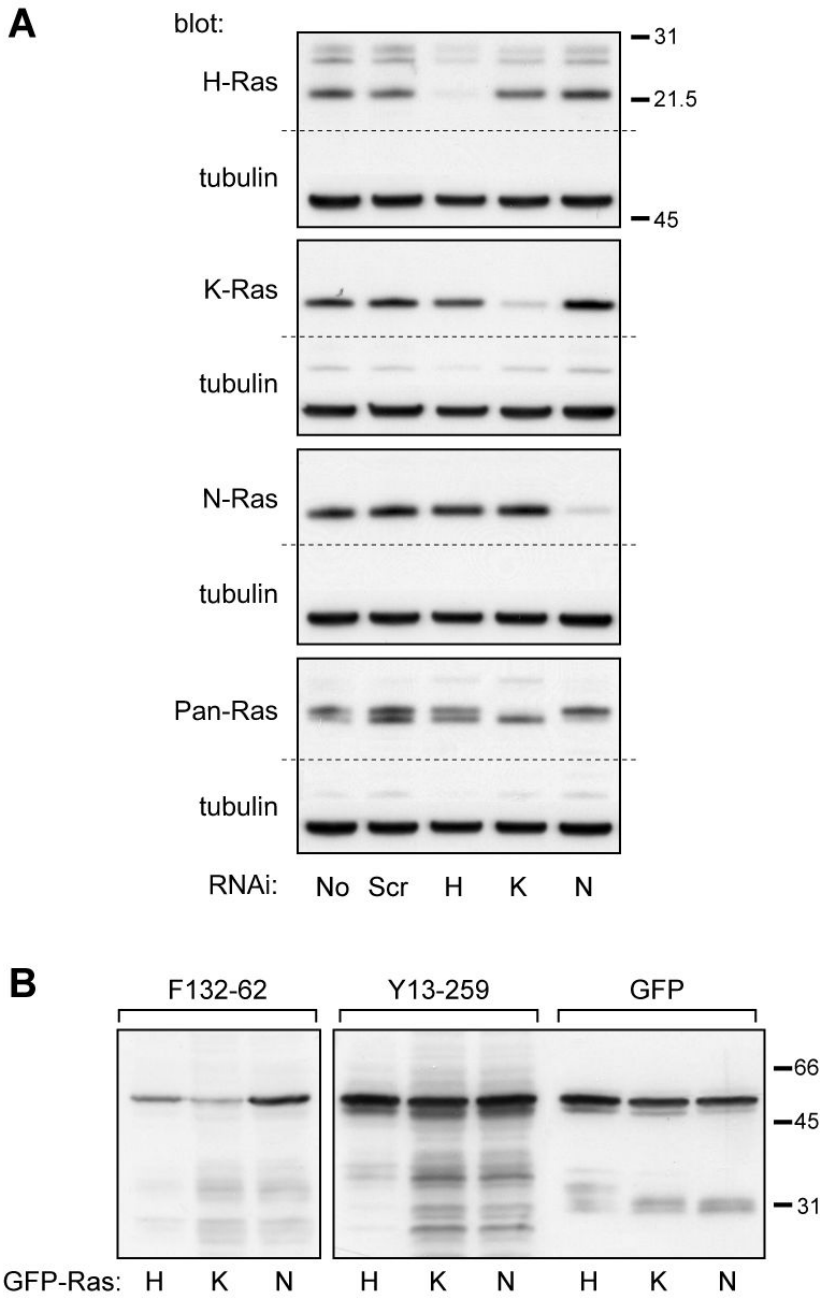

FIG. 2. H-Ras is a minor isoform in HeLa cells compared to K-Ras and N-Ras

(A) Isoform-specific Ras knock-down using RNAi; >90% knock-down of each isoform was achieved. Probing the knock-down lysates with the anti-pan-Ras antibody Y13-259 enables the relative abundance of each isoform to be determined. (B) Y13-259 as a true pan-Ras antibody. Lysates normalised for GFP expression from HeLa cells expressing GFP-tagged Ras isoforms were probed with a variety of anti-pan-Ras antibodies. Only Y13-259 exhibited no isoform preference.

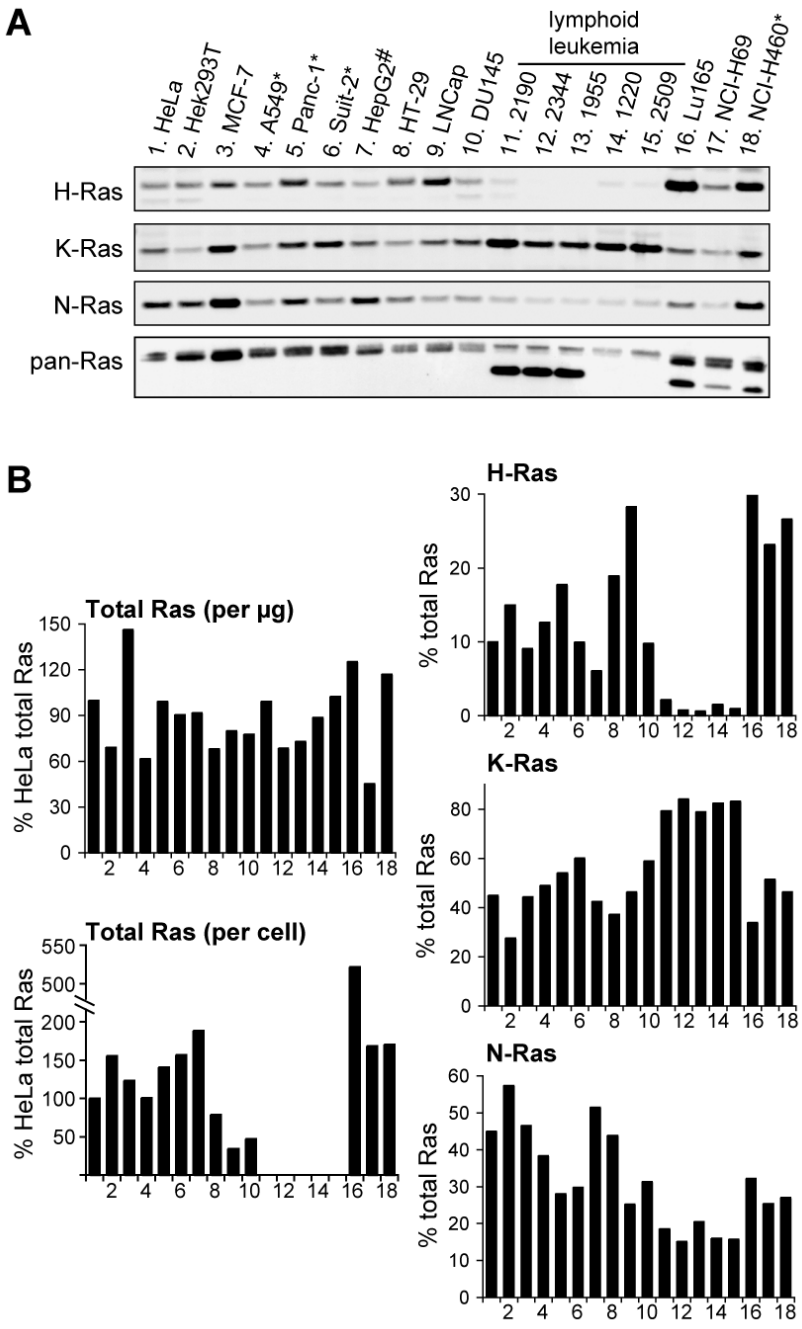

FIG. 3. Quantitative analysis of Ras isoform abundance in cancer cell lines

(A) 10µg of each cell lysate was immunoblotted for Ras isoforms and total Ras (Y13-259). Cell lines harbouring oncogenic K-Ras (*) or N-Ras (#) are highlighted (B); for the leukaemia cells, the Ras mutational status is unknown. HeLa cells were used as a benchmark to determine comparative abundance of Ras isoforms in other cell lines. Primary lymphoid leukaemia samples were only available as lysates preventing determination of total Ras abundance per 1×10^6 cells.

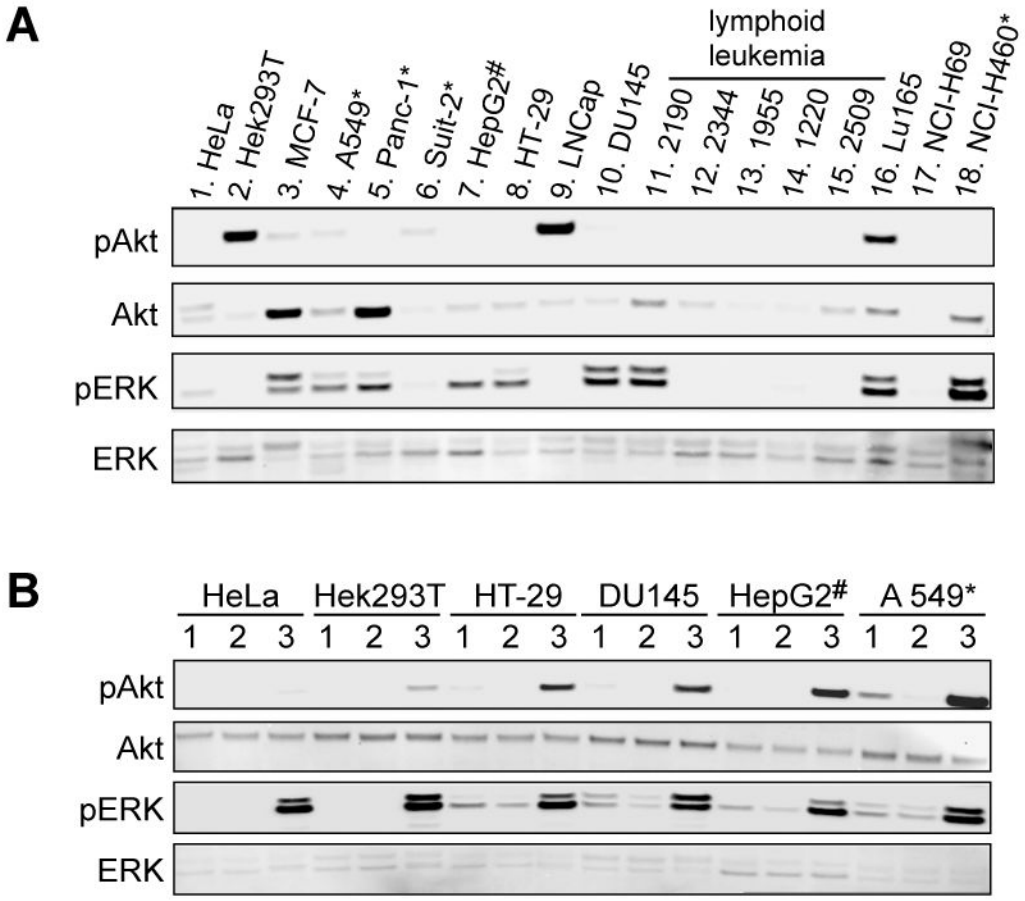

FIG. 4. Oncogenic Ras is largely uncoupled from effector activation

Phospho-Akt and phospho-ERK labelling is not correlated with the presence of oncogenic Ras isoforms (K-Ras,* or N-Ras, #) within cancer cell lines (A). Despite a lack of constitutive stimulation in starved conditions, HepG2 and A549 cells are still able to respond to acute serum stimulation (B). 1: normal 10% serum media overnight, 2: starved overnight, 3: starved overnight then 20% FBS treatment for 5 minutes. Other relevant mutations present in these cell lines include the potent B-Raf V600E (HT-29) and PtdIns-3-kinase p110α E545K (MCF-7 and NCI-H460) oncogenic mutations.

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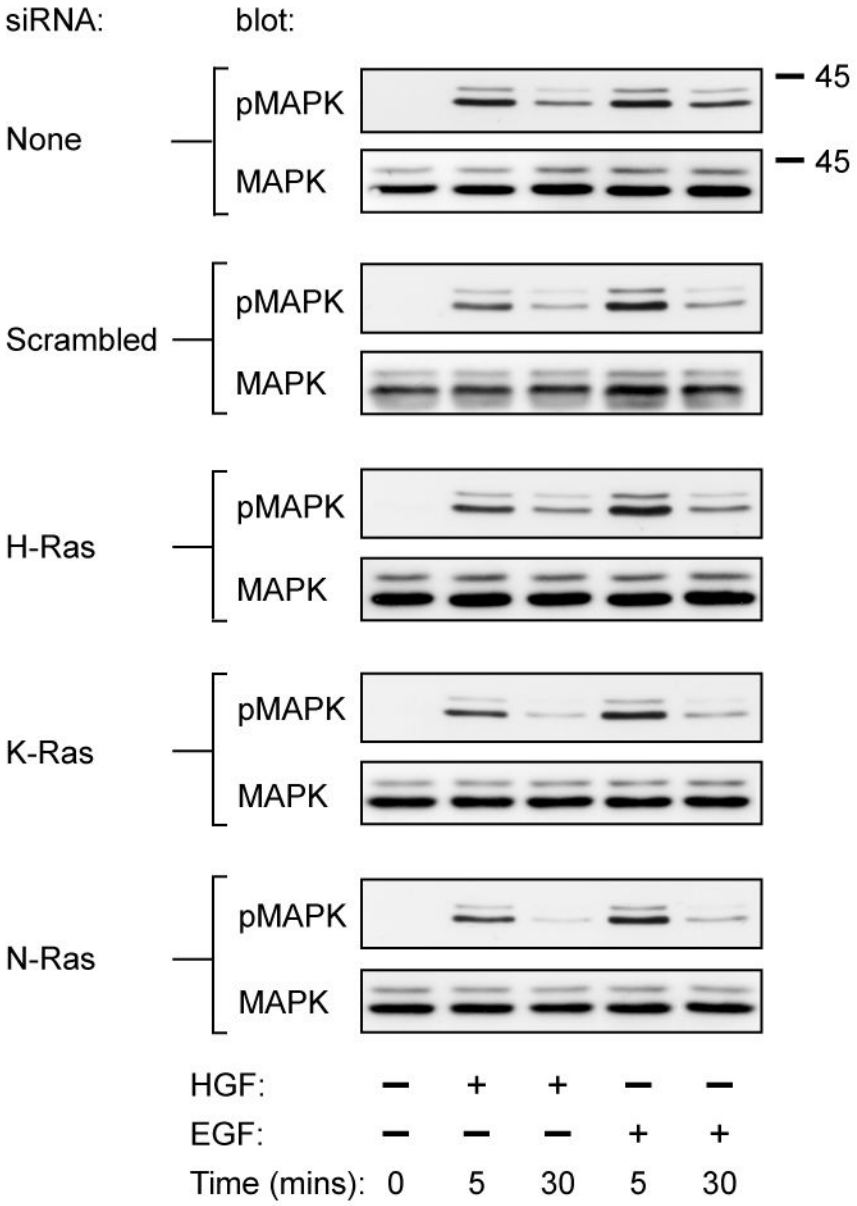

FIG. 5. Ras isoforms are redundant for MAP kinase activation

MAP kinase activation is preserved following Ras isoform-specific knock-down in HeLa cells stimulated with 100ng/ml EGF or HGF. 35μg of each cell lysate was probed with anti-phospho-p44/42 MAPK and anti-p44/42 MAPK antibodies.

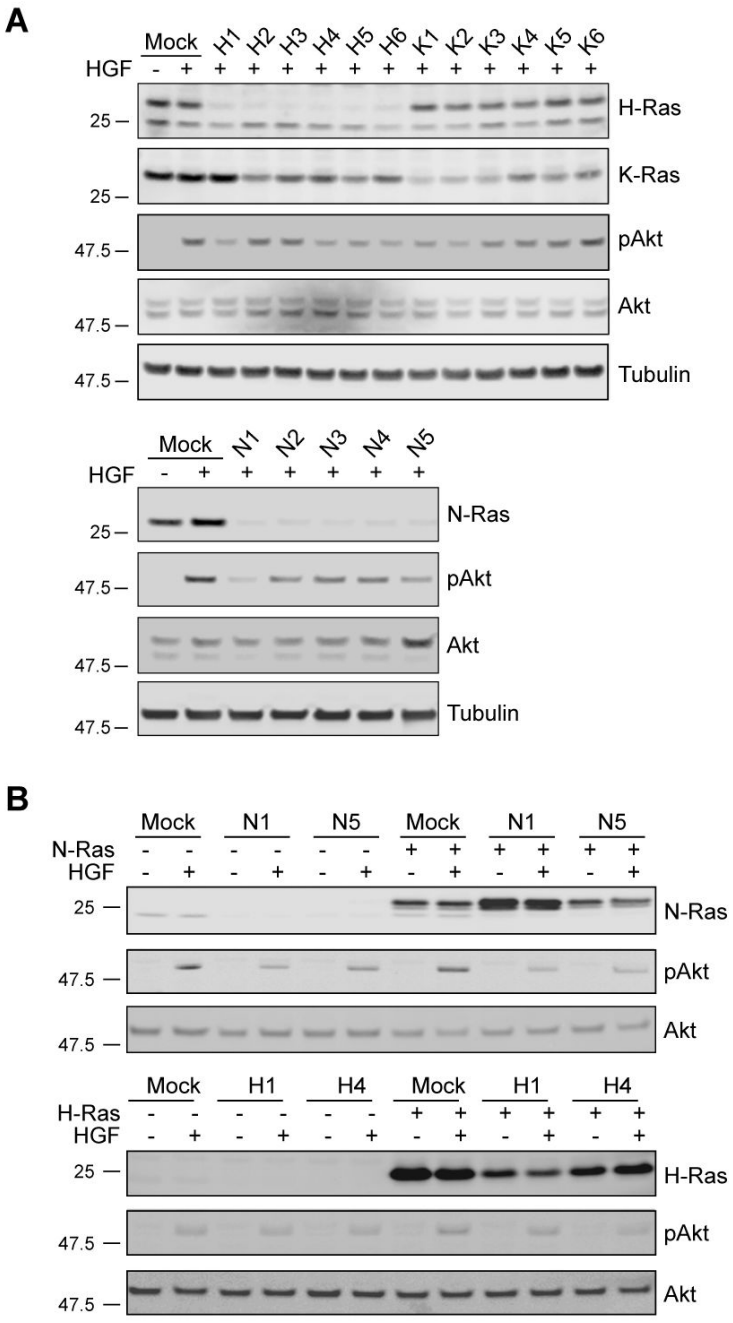

FIG. 6. Ras isoforms are redundant for Akt activation

RNAi treated HeLa cells stimulated with 100ng/ml of HGF were analysed for activating Ser^{473} phosphorylation of Akt (A). Significant abrogation of Akt phosphorylation is seen with some Ras oligos although for H- and N-Ras this doesn't appear to correlate with the extent of Ras knockdown. (B) Recomplementation with RNAi resistant H- or N-Ras fails to fully restore Akt phosphorylation indicating non-specific RNAi effects on Akt signalling.