p204 Protein Is a Novel Modulator of Ras Activity*

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The murine p200 family protein, p204, modulates cell proliferation and tissue differentiation. Many of its activities are exerted in the nucleus. However, in cardiac myocytes, p204 accumulated in the cytoplasm. A yeast two-hybrid assay revealed a p204–cytoplasmic Ras protein interaction. This was confirmed (i) by coimmunoprecipitation of p204 with Ras in mouse heart extract and with endogenous or ectopic H-Ras and K-Ras in cell lysates as well as (ii) by binding of purified H-Ras-GTP to purified p204 in vitro. p204 inhibited (i) the cleavage of RasGTP to RasGDP by RasGAP; (ii) the binding to RasGTP of Raf-1, phosphatidylinositol 3-kinase, and Ral-GDS, effectors of Ras signaling; and (iii) activation by the Ras pathway of the Raf-1, phosphatidylinositol 3-kinase, and Ral-GDS, effectors of Ras signaling; and (iii) activation by the Ras pathway of downstream targets (e.g. MEK, Akt, and p38MAPK). Oncogenic Ras expression triggered the phosphorylation and translocation of p204 from the nucleus to the cytoplasm. This is expected to increase the interaction between the two proteins. Translocation triggered by Ras oncprotein was blocked by the LY294002 inhibitor of phosphatidylinositol 3-kinase. Ras did not promote phosphorylation or translocation to the cytoplasm of mutated p204 in which serine 179 was replaced by alanine. p204 overexpression inhibited the anchorage-independent proliferation of cells expressing RasQ61L oncoprotein. Ras oncprotein triggered in MEF3T3 cells the rearrangement of the actin cytoskeleton and the enhancement of cell migration through a membrane. Overexpression of p204 inhibited both. Ras oncprotein or activated, wild-type Ras was described to increase Egr-1 transcription factor expression. We report that a sequence in the gene encoding p204 bound Egr-1, and Egr-1 activated p204 expression. Ras oncprotein or activated wild-type Ras increased the expression in 3T3 cells of p204 together with that of Egr-1. Furthermore, the activation of expression of a single copy of K-ras oncogene in cultured murine embryonic cells induced the expression of a high level of p204 as well as its distribution between the nuclei and the cytoplasm. Thus, p204 may serve as a negative feedback inhibitor of Ras activity.

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The interferons are vertebrate cytokines with antimicrobial, immunomodulatory, and cell growth and differentiation regulatory activities (1–3). They function by modulating the expression of many genes, including those of the gene 200 cluster (4). In mice, this cluster consists of at least 10 genes that encode the p200 family proteins. The human counterpart of the cluster consists apparently of four genes (MND, IFI16, AIM2, and IFIX) that encode the Hin200 family proteins (5, 6).

Among the best characterized members of the murine p200 family proteins is p202a (which was designated earlier as p202) (7). p202a modulates transcription, cell proliferation, and apoptosis, and its overexpression was correlated with symptoms of lupus erythematosus (8, 9). It functions primarily by binding numerous sequence-specific transcription factors and transcription modulators, including pRb and p53, and inhibiting their activity generally, but not exclusively, by binding them and inhibiting their sequence-specific binding to DNA (8–10).

A second p200 family protein, p205, was originally designated as D3. p205 can also bind pRb and p53. It can inhibit cell proliferation by inducing pRb and increasing the level of p21 in a p53-dependent manner but can inhibit it also independently of pRb and p53 (11).

A third much studied p200 family protein is p204, encoded by the Ifi204 gene. p204 is structurally related to p202a (7). p204 is involved in the modulation of transcription, protein degradation, cell proliferation, and differentiation of numerous tissues. The tissues include, among others, skeletal muscle myotubes (12, 13), heart muscle myocytes (14, 15), bone osteoblasts (16, 17), and macrophages (18). p204 modulates a series of biochemical mechanisms to promote the differentiation of different tissues. Moreover, the expression of p204 in different tissues can be promoted by distinct cytokines, promoters, and tissue-specific transcription factors. Depending on the cell type and stage of differentiation, p204 can be nuclear, nucleoplasmic, and/or cytoplasmic. In performing its various functions, p204 binds and affects the activities of numerous proteins. These include, among others, UBF, the factor involved in ribosomal RNA synthesis (19), the pocket proteins (pRb, p107, and p130) (19, 20) involved in the control of cell proliferation and differentiation, and the Id (inhibitor of differentiation) proteins (13, 15). p204 also occurs in multiprotein complexes (e.g. in the p204–pRb-Cbfα1 complex involved in osteoblast differentiation). In this complex, p204 acts as a transcriptional coactivator (17). p204 was also reported to be required for the replication of cytomegalovirus (21).

Among 10 adult mouse tissues tested, the level of p204 was highest in cardiac myocytes (12). Moreover, in the course of differentiation of the myocytes from embryonal stem cells, the bulk of p204 was translocated from the nucleus to the cyto-
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plasm (12, 14). These facts prompted us to search for cytoplasmic proteins in the cardiac myocytes to which p204 binds. Here we report that cytoplasmic H and K-Ras proteins (which are encoded by the H- and K-ras genes) were bound by p204 and that p204 inhibited the various activities of these multifunctional proteins.

Particular mutants of the ras genes (designated as ras oncopogenes) play a causal role in more than a quarter of human cancers (22–26). Wild-type Ras proteins control signaling pathways. They are GTP-binding proteins, which, when acted upon by specific factors, cycle between an activated and an inactivated form, RasGTP and RasGDP, respectively (27). Ras proteins can be localized on the inner cell membrane or in the Golgi or endosomal compartments (28, 29). They are coupling proteins to downstream signaling pathways regulating transcription, translation, cell growth, proliferation, cell shape, apoptosis, senescence, and malignant transformation (26, 30). ras oncogenes encode Ras oncoproteins, which are persistently activated, since (unlike wild-type Ras proteins) they cannot undergo the GTP-bound state (26, 30).

There are numerous downstream mediator proteins of Ras signaling. These are designated as Ras effectors. They bind to the effector domain of RasGTP (34). These effectors initiate the activation of expression of a single copy of the Ras oncogene (43) in cultured murine embryonic cells induced the expression of high levels of p204 as well as its distribution between the nuclei and the cytoplasm. Thus, p204 might also serve as a negative feedback inhibitor of Ras activity.

EXPERIMENTAL PROCEDURES

Plasmids

pBl-H-RasG12V and pBl-p204 were generated by inserting the appropriate H-RasG12V PCR product or 204 PCR product into the PstI/Sall sites or MuII/NheI sites, respectively, of the pBl plasmid (BD Clontech). The cDNA of the 204 protein (BD Clontech) was fused to the EGFP-C1 vector (BD Clontech), respectively. pCGNHA-K-Ras4B12G was obtained from H. P. Shao. MBP-p204 was purified using amylose affinity chromatography according to the New England Biolabs manual. The purity of His-RasGAP (a kind gift of D. Soll) was verified by PAGE. GST-Raf-1 Ras-binding domain (RBD) was from Upstate Biotechnology, Inc. (Lake Placid, NY). EGFP-GH-RasQ61L and pIRESpuro3-H-RasQ61L were generated by inserting H-RasQ61L cDNA into the EcoRI and BamHI sites of the EGFP-C1 and the pIRESpuro3 vectors (BD Clontech), respectively. pCGNHA-K-Ras4B12G and three other expression plasmids encoding the same protein, except with single amino acid substitutions in the effector segment, were generous gifts from G. J. Clark (45).

pCMV-Egr-1 (46) was from L. Nagy. The pGL-Egr-1 reporter construct was generated by inserting a PCR product extending from nucleotide 6927 to 6647 in the fly204 gene 5′-flanking region from the BAC225 clone (4) between the KpnI and BglII sites of the pGL vector (Promega). The mutant Egr-1 reporter construct (pGL-mEgr-1) was generated using the QuikChange kit (Stratagene). pCMV204 was produced by S. J. (47).

Fusion Proteins

A plasmid encoding the MBP-p204 fusion protein was expressed in Escherichia coli BL21-Gold(DE3)PlysS codon plus (kindly provided by D. Soll). MBP-p204 was purified using amylose beads (New England Biolabs). Its purity was verified by 4–20% PAGE and Coomassie Brilliant Blue staining according to the New England Biolabs manual. The purity of His-RasGAP (a kind gift of T. Koleske) was also verified by PAGE. GST-H-Ras expressed in E. coli DH5α was purified on a GST column (see Fig. S2).

Interferon

A modified human α-interferon active in murine cells was a kind gift of C. Weissmann (47).

Antibodies

Antibodies to H-Ras and K-Ras were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies to p204 were described (19). Anti-MBP was from New England Biolabs; anti-HA was from Roche Applied Science; anti-MEK, anti-phospho-MEK, anti-Akt, anti-phospho-Akt, anti-p38, and

The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; CaMK, calcium/calmodulin-dependent protein kinase; Con, control; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; FCS, fetal calf serum; GST, glutathione S-transferase; HA, hemagglutinin; Id, inhibitor of differentiation; MAPK, mitogen-activated protein kinase; MBP, maltose-binding protein; MEF, murine embryo fibroblast; MEK, mitogen-activated protein extracellular signal-regulating kinase; NES, nuclear export signal; NLS, nuclear localization signal; PDGF, platelet-derived growth factor; RBD, Ras binding domain; RasGAP, enzyme cleaving RasGTP to RasGDP; GTPγS, guanosine 5′-3-(O-thio)triphosphate; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; N, N-terminal.

3 The abbreviations used are: PI3K, phosphatidylinositol 3-kinase; CaMK, calcium/calmodulin-dependent protein kinase; Con, control; DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; FCS, fetal calf serum; GST, glutathione S-transferase; HA, hemagglutinin; Id, inhibitor of differentiation; MAPK, mitogen-activated protein kinase; MBP, maltose-binding protein; MEF, murine embryo fibroblast; MEK, mitogen-activated protein extracellular signal-regulating kinase; NES, nuclear export signal; NLS, nuclear localization signal; PDGF, platelet-derived growth factor; RBD, Ras binding domain; RasGAP, enzyme cleaving RasGTP to RasGDP; GTPγS, guanosine 5′-3-(O-thio)triphosphate; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein; DAPI, 4′,6-diamidino-2-phenylindole; N, N-terminal.
anti-phospho-p38 from Cell Signaling; anti-Egr-1 from Santa Cruz Biotechnology; anti-β-actin and anti-FLAG from Sigma; and anti-β-tubulin from the Developmental Studies Hybridoma Bank.

Cell Cultures

Cells of the human embryonic HEK293; the murine AKR-2B, MEF3T3-Tet-off, NIH3T3, BLK, FT9, NB490, NB508 (43, 48), 11.7+Cre, 11.7-Cre and 11.1Wt+Cre (43, 48); and the monkey COS-7 lines were maintained in DMEM, 10% FCS medium and 11.7% CO2 at 37 °C.

Generation of Stable Cell Lines

A stable cell line with a low level of constitutive H-Ras expression was generated by transfection of the pIRESpuro3-H-RasQ61L plasmid with Lipofectamine 2000 into an AKR-2B line in which p204 was inducible by edcysone or ponasterone A (19) and selection with 6 μg/ml puromycin in DMEM, 10% FCS for 2 weeks. A clone expressing upon induction a low level of H-RasQ61L was picked. A control cell line was generated by transfection of the vector. The doxycycline-inducible lines MEF3T3-Tet-off-H-RasQ61L, MEF3T3-Tet-off-p204, and MEF3T3-Tet-off-H-RasQ61L_p204 were obtained by transfection into the MEF3T3-Tet-off line (Clontech) of pB1-H-RasQ61L and/or pB1-204, together with a hygromycin resistance plasmid, followed by selection with hygromycin.

Coimmunoprecipitation (for Fig. 2, B–D)

FT9 cells (i.e. NIH3T3 cells expressing an oncogenic H-Ras mutant) (49) were maintained in DMEM, 10% FCS medium and harvested in MLB lysis buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Igepal CA-630 (Sigma), 0.25% sodium deoxycholate, 10% glycerol, 10 mM MgCl2, 1 mM EDTA, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Lysate aliquots with 500 μg of total protein per 0.5 ml were incubated with protein G beads with conjugated anti-H-Ras (Upstate Biotechnology, Inc.) or anti-p204 antibodies at 4 °C overnight. The beads were washed and boiled in SDS loading buffer, and the eluate was subjected to 4–20% SDS-PAGE for Western blotting, using antibodies against H-Ras, K-Ras, or p204. Similar procedures were used in the coimmunoprecipitation experiments in Fig. 2, C and D, which involved different cell lines from that used in the experiments shown in Fig. 2B.

Assay of RasGTP/GDP Binding to p204 (for Fig. 2F)

Cells of the MEF3T3-Tet-off-p204 line were plated in 20 10-cm dishes and, for inducing p204, were incubated first with 1 μg/ml doxycycline for 24 h. Then the doxycycline was removed, and the cultures were incubated for a further 48 h. The cultures were lysed in MLB lysis buffer, and aliquots from the lysate were incubated with GTPγS or GDP at 30 °C for 30 min. Ras protein was pulled down, using protein A beads conjugated with anti-p204 and, as a positive control, beads to which GST-Raf-1 RD was conjugated.

For Fig. 3, pcDNA3–204 or pcDNA3–204 segment expression plasmids were generated by inserting 204 cDNA or its various segments (prepared by PCR) between the EcoRI and BamHI sites of the vector. The plasmids were expressed in reticulocyte lysate (Promega) in the presence of [35S]methionine. 10-μl aliquots from each [35S]-labeled segment were added to 0.5-ml aliquots containing 500 μg of protein from a lysate of cultured HEK293 cells that had been transfected with the expression plasmid pCGN-HA-H-RasQ61L and preincubated with GTPγS. The reaction mixture was incubated at 4 °C overnight. The labeled p204 or its various segments bound to HA-H-RasQ61L-GTPγS were pulled down with protein G/A beads loaded with anti-HA antibodies and assayed by SDS-PAGE and autoradiography.

For Fig. 4, the effects of MBP-p204, and, as a control, MBP, on RasGAP activity were assayed in vitro, using the RasGAP assay (50). Purified GST-H-Ras, His-RasGAP, MBP-p204, and MBP were used (see Fig. S2). The [α-32P]GTP and [α-32P]GDP nucleotides were separated by thin layer chromatography on polyethyleneimine-cellulose plates (Cel 300 PEI TLC; Sorrent Technology) (51) and assayed with a PhosphorImager analyzer.

Assays of the Inhibition by p204 of the Binding of H-Ras to Raf-1, PI3K, and RalGDS (for Fig. 5)

Raf-1—MBP and MBP-p204 fusion protein were purified using amylose beads (New England Biolabs) and were quantified by the Bio-Rad protein assay (see Fig. S2). NIH3T3 cells were incubated in DMEM, 0.5% FCS for 48 h, and, if so indicated, the level of RasGTP was increased in them by adding 100 ng/ml EGF to the medium for the last 10 min. MBP-p204, MBP, or neither was incubated with GST-Raf-1 RD (Upstate Biotechnology) immobilized on agarose beads at 4 °C for 30 min and then added to the lysates of the NIH3T3 cells and further incubated at 4 °C for 30 min. The beads were washed and eluted with boiling SDS loading buffer, and the H-Ras eluted was assayed by Western blotting, following the Upstate Biotechnology protocol.

PI3K—HEK293 cells were transfected with pIREs-H-RasQ61L or with pcDNA3P110α-HA and pcMV6-P85α-FLAG, using Lipofectamine 2000, and incubated in DMEM, 10% FCS medium in 10% CO2 for 48 h. The cells were harvested and lysed in MLB buffer. Total protein levels were assayed and adjusted to 500 μg of protein/0.5 ml of lysate. If so indicated, 400 nmol of MBP or 400 of nmol MBP-MBP-204 were incubated with 0.5 ml of lysate of cells expressing H-RasQ61L at 4 °C for 30 min and were then supplemented with 0.5 ml of a lysate from cells expressing P110α-HA and P85α-FLAG and incubated with protein G beads loaded with anti-HA antibodies or mouse IgG control at 4 °C overnight. The beads were washed and eluted with boiling SDS loading buffer. 30-μl samples were analyzed by Western blotting, using anti-H-Ras antibodies.

RalGDS—A culture of HEK293 cells was infected with retrovirus-packaged pBabepro-Myc-RalGDS. A second culture of HEK293 cells was transfected with pCGN-HA-H-RasQ61L. After incubation, cell lysates were prepared. The lysate from the culture transfected with pCGN-HA-H-RasQ61L was incubated, as indicated, without or with MBP or MBP204. These incubated lysates were mixed with the lysate from the culture infected with retrovirus-packaged pBabepro-Myc-RalGDS and processed by pull-down with immobilized Myc

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antibodies or mouse IgG and Western blotting, using anti-HA.

Assay of the Inhibition by p204 of the Phosphorylation of MEK, Akt, and p38MAPK

For Fig. 6A, an AKR-2B line, in which p204 expression was induced by ponasterone A (AKR-2B-Ind-p204) and a control line (AKR-2B-Ind-Con) were established from AKR-2B cells by transfection of constructs encoding ponasterone A receptors and selectable markers from the ecdysone-inducible expression kit (Invitrogen) as well as inducible p204, followed by selection (12). To induce p204, the cells were incubated with 1 μM ponasterone A in DMEM, 0.5% FCS for 48 h. EGF (200 ng/ml) was added 30 min before harvesting the cells in SDS loading buffer. 25-μl aliquots of the lysates were subjected to Western blotting with p204 antibodies. Phosphorylation of MEK triggered by EGF (via the Ras/Raf-1 pathway) as well as the amount of total MEK protein were assayed by Western blotting, using antiphospho-MEK and anti-MEK antibodies. The levels of phospho-Akt, total Akt, phospho-p38MAPK, total p38MAPK, and p204 proteins were similarly assayed after stripping the membranes and applying appropriate antibodies.

For Fig. 6B, one NB508 culture was transfected with a pCMV204AS expression plasmid, a second was transfected with the pCMV vector, and both cultures were also transfected with a hygromycin resistance expression plasmid. After selection with hygromycin, resistant clones were selected. These were incubated in DMEM, 10% fetal bovine serum for about 48 h. When reaching 80% confluence, the culture was washed with cold phosphate-buffered saline containing 1 mM NaF and 1 mM Na3VO4 and lysed in SDS loading buffer. Aliquots containing 60 μg of protein were subjected to Western blotting.

Assay of the Growth Inhibition by p204 in Monolayer Cultures

(for Fig. 7B)

The Con, Ind-p204, and H-RasO61L-Ind-p204 lines were plated at 3 × 105 cells/ml in 60-mm tissue culture dishes in growth medium, and, if so indicated, 1 μM ponasterone A was added. The cultures were incubated for 72 h. After digestion with trypsin-EDTA, the cells were counted, using a microscope.

Assays of Anchorage-independent Growth in Soft Agar Were Performed as Described (52) (for Fig. 7, C and D)

In brief, the bottom agar layer contained 0.51% agar (Bacto-Agar; Difco) in DMEM (Invitrogen), 10% fetal bovine serum. The upper agar layer contained 0.34% agar in DMEM, 13.3% fetal bovine serum. If indicated, 4 μM ponasterone A was added to the upper layer. AKR-2B-Ind-p204 or AKR-2B-H-RasO61L-Ind-p204 were plated in the upper layer at 104 cells/60-mm dish. Triplicate cultures were prepared for each cell type and treatment. Cells were cultured in 5% CO2 in a 37 °C incubator for 2 weeks and photographed.

Immunofluorescent Staining (for Fig. 9)

MEF3T3-Tet-off-H-RasG12V, MEF3T3-Tet-off-p204, or MEF3T3-Tet-off-H-RasG12V-p204 cell lines were incubated in the absence or presence of 20 μM PD98059 or LY294002 dissolved in

Me2SO or of Me2SO (serving as a control) in the absence of doxycycline for 36–48 h. MEF3T3-Tet-off-H-RasG12V cells were transfected with pCMV204 or pCMV204S179A and incubated in the absence of doxycycline for 48 h to induce H-RasG12V. The cells were fixed and processed, using 3.7% formaldehyde with Alexa Fluor 555 phalloidin (Invitrogen), anti-p204 antibodies, and fluorescein isothiocyanate-tagged anti-rabbit IgG, as indicated in the figures.

Chromatin Immunoprecipitation (for Fig. 11A (b))

The chromatin was cross-linked with 3% formaldehyde. The pGL-Egr-1 reporter gene (in Fig. 11A (d)) was constructed by inserting a 453-bp PCR product (extending from nucleotide −6927 to −6474 in the Ifi204 gene 5′-flanking region) into the pGL3 vector. A control reporter gene with a mutated Egr-1 binding sequence (pGL-mEgr-1) was constructed, using the QuickChange kit. T3T cells were cotransfected with the plasmids specified. Luciferase activity was assayed after a 36-h incubation.

Mouse Embryonic Fibroblasts (MEFs) (for Fig. 12)

MEFs were prepared from LSL-KrasG12D mice (43) and littermate controls. In brief, 13.5-day-old embryos were isolated, minced with razor blades, trypsinized, and cultured as described previously (48). Cre recombinase was introduced into MEFs via retrovirus-mediated gene transfer of Cre-GFP. Infected cells were isolated by fluorescence-activated cell sorting. Cre-mediated deletion of the STOP cassette in LSL-KrasG12D was confirmed by PCR.

RESULTS

The Bulk of p204 Is Cytoplasmic in the Heart; Cytoplasmic Proteins Binding to p204—In mouse embryos, the level of p204 was low in the heart on embryonic day 10.5, and it greatly increased until birth (12). This high p204 level was maintained for at least 5 months (Fig. 1A). In tissue slices
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from hearts of 3-month-old mice, the bulk of p204 was cytoplasmic (Fig. 1B). The translocation of p204 from the nucleus to the cytoplasm depends on a nuclear export signal (NES) in p204, and it takes place in the course of the differentiation of embryonal carcinoma stem cells to cardiac myocytes (14).

The proteins known to be bound by p204 at present are primarily nuclear. Wishing to identify cytoplasmic proteins interacting with p204, we performed a yeast two-hybrid assay using a cDNA library from hearts of 8–12-week-old male mice. The assay revealed numerous cDNA clones encoding proteins interacting with p204. Many of these proteins were related to the cytoplasmic H-Ras protein (not shown).

Binding of H-Ras and K-Ras to p204—Testing the validity of the results of the two-hybrid assay, we performed a series of coimmunoprecipitation and pull-down assays (Fig. 2). Immunoprecipitation of a heart extract from 3-month-old mice with anti-p204 resulted in the coimmunoprecipitation of the endogenous H-Ras, together with the endogenous p204 (Fig. 2A). Immunoprecipitation with anti-H-Ras coprecipitated p204, and immunoprecipitation with anti-p204 coprecipitated H-Ras from a lysate of FT9 cells (Fig. 2B).

These cells were generated by transfection of approximately six copies of the H-ras oncogene into NIH3T3 cells (49). The experiments in Fig. 2C were performed using two murine primary adenocarcinoma cell lines (NB490 and NB508), which were established from freshly isolated tumor specimens (53, 54). The generation of the tumors in mice involved the activation of expression at the endogenous level of a latent K-RasG12D knock-in allele (LSL-K-Ras) by Cre-mediated excision of a transcriptional stopper element (43). As revealed by microarray-based comparative genomic hybridization, both lines should have nearly physiological levels of K-Ras expression. Line NB490 appears to have a single extra gene copy of either the K-RasG12D allele or of wild-type K-Ras, and NB 508 has the wild-type gene copy number.7 D, binding of p204 to K-RasG12V and to three different mutants of K-RasG12V in the K-Ras effector binding region (kindly provided by G. J. Clark). Plasmids encoding K-RasG12V and the three mutants were transfected into different NIH3T3 cultures. Coimmunoprecipitation from culture lysates with anti-K-Ras antibodies conjugated to protein G beads and Western blotting with anti-p204 antibodies. E, binding of purified MBP-p204 to purified GST-K-RasG12V-GTPyS. The pull-down assay used immobilized anti-MBP antibodies. GST-H-RasG12V and MBP-p204, both from bacterial lysates, were purified on GST-beads and MBP-beads, respectively. GST-H-RasG12V-conjugated beads were first incubated with GTPyS and subsequently with MBP-p204 (which was eluted and concentrated from MBP beads) at 4°C overnight. The pulled down MBP-p204 was assayed by Western blotting with anti-MBP antibodies. As negative controls, MBP fused to Paramyosin, MBP, and MBP-p204 incubated with GST-beads were used. F, binding of H-Ras-GTPyS to induced endogenous p204. Shown is a pull-down assay with immobilized anti-p204 antibodies and Western blotting with anti-H-Ras antibodies. Bottom, p204 was induced in a MEF3T3-Tet-off-p204 culture by incubation in DMEM without doxycycline. Western blotting is shown. Top, cultures were incubated without doxycycline to induce p204 and lysed, and aliquots from the lysate were incubated with GTPyS or GDP. For the pull-down assay, aliquots were incubated as specified with immobilized anti-p204 antibodies (or as a negative control with immobilized mouse IgG). As positive controls for the pull-down assay, immobilized Raf-1 RBD was used. The pulled down H-Ras was detected by Western blotting with anti-H-Ras antibodies. For further details, see "Experimental Procedures.”

FIGURE 2. Binding of endogenous and ectopic p204 to endogenous and ectopic murine wild type and mutant activated Ras proteins. Coimmunoprecipitation and pull-down assays are shown. A, coimmunoprecipitation of H-Ras and p204 from the extracts of hearts of 3-month-old C129 mice. Hearts were washed, their ventricles were excised and disintegrated in MLB lysis buffer, and the debris was sedimented by centrifugation. Anti-p204 antiserum was used for immunoprecipitation from the supernatant fraction (5 mg of protein), and anti-H-Ras antibodies were used for Western blotting. Preimmune IgG was used as a negative control. B, coimmunoprecipitation of endogenous p204 with endogenous H-Ras in lysates of cells from the murine FT9 line that was generated by transfection of NIH3T3 cells with a plasmid encoding activated H-RasG12V. Top, coimmunoprecipitation with anti-H-Ras antibodies immobilized on protein A and Western blotting with anti-p204 antibodies. Bottom, coimmunoprecipitation with anti-p204 antibodies immobilized on protein A and Western blotting with anti-H-Ras antibodies in both panels, preimmune IgG was used as a negative control, and the amounts of input p204 and H-Ras are indicated. C, the NB490 and NB508 mouse tumor cell lines (kindly provided by N. Bardeesy) were cultured in DMEM, 10% FCS. If so indicated (+) 1000 units/ml α-interferon was added for 48 h. Coimmunoprecipitation from the culture lysates was performed with anti-K-Ras antibodies conjugated to protein G beads and Western blotting with anti-p204. As a negative control, preimmune IgG was used. As determined by microarray-based comparative genomic hybridization, the NB490 cells appear to have a
copy of either the K-RasG12D allele or of wild-type K-Ras, and line NB508 has the wild-type gene copy number.4

Immunoprecipitation with anti-K-Ras (but not with preimmune IgG) of lysates from NB508 and NB490 cells coimmunoprecipitated p204 (Fig. 2C). Treatment with α-interferon increased the level of endogenous p204 only in the NB490 line. This resulted in an increase of the amount of p204 coimmunoprecipitated by anti-K-Ras. Anti-K-Ras also coimmunoprecipitated p204 from a lysate of NIH3T3 cells expressing ectopic K-RasG12V oncoprotein (Fig. 2D). Purified p204 (actually MBP-p204) could be pulled down by immobilized, purified H-Ras (actually GST-H-RasG12V) (Fig. 2E). This established that the binding of p204 to H-Ras did not require the involvement of further proteins.

p204 was bound to H-RasGTP (the activated form of H-Ras) but not to H-RasGDP (Fig. 2F, top). In the MEF3T3 cells, serving as the source of p204, endogenous p204 was induced by removal of doxycycline from the medium (Fig. 2F, bottom). As a positive control, the Ras-binding domain of the Raf-1 protein (Raf-1 RBD) was used (Fig. 2F, top). Raf-1 RBD is known to bind to RasGTP but not to RasGDP.

Mapping the Regions in p204 That Bind H-Ras—Aliquots of HA–H-RasG12V loaded with GTP·S (a GTP derivative that is resistant to cleavage by RasGAP) were incubated with p204 (Fig. 3A) or its various segments (Fig. 3, B–F) labeled with [35S]methionine. Thereafter, the HA–H-RasG12V · GTP·S was pulled down by immobilized anti-HA antibodies and the associated labeled p204 or its segments were assayed by SDS-PAGE and autoradiography. The band patterns (Fig. 3, A–F) revealed the binding of p204 segments to immobilized HA-H-RasG12V (Fig. 3, top). The bands in the A–F lanes correspond to the A–F p204 segments in the top, N-terminal segment; a and b, two 200-amino acid long homologous segments; c, C-terminal segment. For further details, see “Experimental Procedures.”

Mapping the Regions in p204 that Bind H-RasQ61L—Aliquots from a cell lysate, including HA-tagged H-RasG12V (HA–H-RasG12V), which had been preincubated with GTP·S (for conversion to HA–H-RasG12V · GTP·S), were further incubated, as indicated, with [35S]methionine-labeled p204 (A) or p204 segment (B–F). The labeled p204 or its segment bound to HA–H-RasG12V · GTP·S was pulled down with immobilized HA antibodies and assayed by SDS-PAGE and autoradiography (bottom). The bands in the A–F lanes in the bottom correspond to the A–F p204 segments in the top. N, N-terminal segment; a and b, two 200-amino acid long homologous segments; c, C-terminal segment. For further details, see “Experimental Procedures.”

**Figure 3.** Mapping of regions in p204 that bind H-RasG12V. Aliquots from a cell lysate, including HA-tagged H-RasG12V (HA–H-RasG12V), which had been preincubated with GTP·S (for conversion to HA–H-RasG12V · GTP·S), were further incubated, as indicated, with [35S]methionine-labeled p204 (A) or p204 segment (B–F). The labeled p204 or its segment bound to HA–H-RasG12V · GTP·S was pulled down with immobilized HA antibodies and assayed by SDS-PAGE and autoradiography (bottom). The bands in the A–F lanes in the bottom correspond to the A–F p204 segments in the top. N, N-terminal segment; a and b, two 200-amino acid long homologous segments; c, C-terminal segment. For further details, see “Experimental Procedures.”

**Figure 4.** p204 Inhibited the activity of RasGAP. Assay by thin layer chromatography and PhosphorImager. Top, purified GST-H-Ras was loaded with [α-32P]GTP. Subsequently, if so indicated, purified His-RasGAP and/or MBP-p204 and/or MBP (serving as control) were added to the reaction mixture in the amounts specified. After incubation, [α-32P]GTP and [α-32P]GDP was separated by thin layer chromatography, and the chromatogram was examined, using a PhosphorImager analyzer. Bottom, diagram indicating the ratios of [α-32P]GTP to [α-32P]GDP in the reaction mixtures 1–6. For further details, see “Experimental Procedures.”

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4 N. Bardeesy, personal communication.
p204 Protein Is a Novel Modulator of Ras Activity

Ras might include the "effector loop" (55). The conformation of this loop is different in RasGTP from that in RasGDP.

The various downstream signaling pathways activated by Ras are initiated by "effector" proteins. The earliest identified of these proteins are Raf-1, PI3K, and Ral-GDS (37-41). The effector proteins bind to RasGTP but not to RasGDP. The regions in RasGTP to which the effector proteins bind include the effector loop (34). These considerations prompted us to test whether p204 (shown in Fig. 2F) also binds to RasGTP and inhibit the binding of the effector proteins to RasGTP.

As expected, the binding of wild-type H-Ras to immobilized RasGTP increased in a lysate from serum-starved cells if the cells were incubated with EGF prior to their lysis (Western blotting in Fig. 5A). This incubation with EGF is known to promote the replacement of the GDP bound to Ras by GTP (56).

Adding purified p204 (actually MBP-p204) strongly decreased the amount of H-Ras bound to GST-Raf-1 RBD in a lysate from cells exposed to EGF, whereas the addition of a purified MBP control had no such effect. These results indicated that p204 inhibited the binding of the Raf-1 RBD domain to RasGTP (Fig. 5A).

In the experiments in Fig. 5, B and C, permanently activated Ras oncoprotein, i.e. H-RasQ61L, was used. This oncoprotein is incapable of cleaving the GTP it binds to GDP. The inhibition by p204 of the binding to RasGTP of a second Ras effector, PI3K, was tested in Fig. 5B. This effector consists of a catalytic subunit (P110α forming a heterodimer with a regulatory subunit (p85α)). We used an HA-tagged version of P110α, allowing its immunoprecipitation with anti-HA antibodies. The data obtained revealed that purified MBP-p204 (but not MBP) inhibited the binding of H-RasGTP to PI3K.

The data in Fig. 5C revealed that the binding of HA-tagged, activated H-RasQ61L to a third Ras effector (i.e. Ral-GDS) (tagged with Myc) was also inhibited by MBP-p204. As a negative control for the specific antibodies used, we established that mouse IgG did not pull down PI3K-H-RasGTP (Fig. 5B), or Ral-GDS-H-RasGTP (Fig. 5C). These data revealed that the binding of p204 to RasGTP resulted in the inhibition of the binding of each of the above tested three Ras effectors to RasGTP.

As noted earlier, the regions in RasGTP to which the effector proteins bind include the effector loop. We tested by coimmunoprecipitation with anti-H-Ras antibodies used, we established that mouse IgG did not pull down PI3K-H-RasGTP (Fig. 5B), or Ral-GDS-H-RasGTP (Fig. 5C). These data revealed that the binding of p204 to RasGTP resulted in the inhibition of the binding of each of the above tested three Ras effectors to RasGTP.

As noted earlier, the regions in RasGTP to which the effector proteins bind include the effector loop. We tested by coimmunoprecipitation...
The weaker binding of p204 to this mutant than to wild-type H-Ras is probably due in part to the fact that the mutant’s affinity to GTP is weaker than that of wild-type H-Ras (and as shown in Fig. 2F, p204 binds to RasGTP).

**An Increase in p204 Level Inhibited, whereas a Decrease in Endogenous p204 Level Enhanced the Phosphorylation of the MEK, Akt, and p38MAPK Mediators of Ras Signaling Pathways.**—We explored whether the inhibition by p204 of the binding of the Ras effectors to RasGTP (Fig. 5) inhibited the signaling through the pathways initiated by the effectors tested. The results in Fig. 6 revealed that this was the case. The experiments involved cells from a line (AKR-2B Ind-p204) in which the level of endogenous p204 could be induced by ponasterone A (Fig. 6, left). The level of p204 induced by ponasterone A was physiological (i.e. not higher than that obtained by induction with the physiological p204 inducer α-interferon) (Fig. 6A, two left bottom panels of Western blotting and the bottom right diagram). As a control, we used a cell line (AKR-2B Ind-Con cells) in which the level of p204 was unaffected by ponasterone A (Fig. 6, right). To increase the RasGTP level in the cells, they were serum-starved and then exposed to EGF for 10 min prior to testing the levels of the various proteins by Western blotting.

As shown in Fig. 5A, p204 inhibited the binding to RasGTP of the Ras effector Raf-1 kinase RBD. When not bound to RasGTP, Raf-1 kinase is cytosolic and inactive. RasGTP is attached to the cell membrane, thus, by binding to it, Raf-1 kinase also becomes membrane-attached. This results in its phosphorylation and the activation of its kinase. The target of the activated Raf-1 kinase that it phosphorylates is MEK, which is converted to phospho-MEK (dual specificity protein kinase). The two top panels of Western blots and the top diagram of Fig. 6A revealed that the inhibition of the binding of Raf-1 kinase to RasGTP by p204 decreased the extent of phospho-MEK formation by ~40% without affecting the level of total MEK protein.

The Western blots also revealed that in cells not exposed to EGF no formation of phospho-MEK was detected. This was presumably in consequence of the very low level of RasGTP in the serum-starved cells (compare the leftmost and the middle panels in the right Western blots assayed with anti-phospho-MEK).

The second Ras effector, whose binding to RasGTP was shown to be inhibited by p204 in Fig. 5, is PI3K (39). When activated by binding to RasGTP, PI3K phosphorylates a phospholipid (i.e. a membrane-embedded phosphatidylinositol-diphosphate). The product of this phosphorylation, the membrane-associated phosphatidylinositoltriphosphate, attracts a serine-threonine kinase designated as Akt (or PKB). The binding of Akt to the phosphatidylinositoltriphosphate moiety results in the phosphorylation of Akt (i.e. phospho-Akt) (59). As shown in Fig. 6A, the formation of phospho-Akt decreased in

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**FIGURE 6.** An increase in p204 level inhibits, whereas a decrease in endogenous p204 level enhances, the phosphorylation of the MEK, Akt, and p38MAPK mediators of Ras signaling pathways. A (left), in the AKR-2B Ind-p204 cell line (but not in the control AKR-2B Ind-Con line) ponasterone A induced the expression of p204 (compare 1, 2, 3, and 4 in the anti-p204 panel). Cells from both lines were serum-starved and, if so indicated, were exposed to ponasterone A for 48 h, and to EGF for 30 min before lysing the cells in SDS loading buffer and determining the levels of MEK, Akt, p38MAPK, phospho-MEK, phospho-Akt, phospho-p38MAPK, and p204 by Western blotting with appropriate antibodies. The level of p204 was also assayed as above in an AKR-2B Ind-p204 culture exposed to α-interferon. Bottom left panel, the Western blots were scanned and graphed using ImageQuant 5.2 Excel software and are shown in the right diagrams. The bar numbers in the right diagrams correspond to the numbers below the columns of the Western blots. The two lowest panels compare the levels of p204 in AKR-2B Ind-p204 cells exposed to α-interferon (2) with that in the same type of cells but exposed to 1000 units/ml α-interferon (5); the latter was taken as 1. B, NB508 murine primary adenocarcinoma cells contain a single copy of K-RasG12D allele and a single copy of wild-type K-Ras allele. One NB508 culture (204AS) was transfected with a pCMV204AS plasmid (encoding 204 antisense RNA), whereas a control NB508 culture (Con) was transfected with a pCMV vector. Stably transfected cell clones were generated after selection with hygromycin. After incubation, cells were harvested, and the levels of p204, phospho-MEK, total MEK, phospho-Akt, and total Akt were determined in the cell lysates by Western blotting with appropriate antibodies (left). The Western blots were scanned and graphed, and the diagrams are shown (right). S.D. values are indicated. For further details, see “Experimental Procedures.”
consequence of the inhibition of the binding of PI3K to RasGTP by p204 by about 40% with no decrease in the level of total Akt protein.

Fig. 6A revealed that the induction of p204 also inhibited the phosphorylation of a further protein, p38MAPK, by about 50% without affecting the total level of p38MAPK protein. p38MAPK is also among the mediators of ras oncogene action. It was reported to be phosphorylated and activated in H-ras oncogene-transformed MCF10A human breast epithelial cell lines in consequence of the cooperative functioning of the PI3K and Rac pathways (60). p38MAPK can, however, also be activated by tumor necrosis factor in endothelial cells by a complex pathway involving, among others, AIP (a RasGAP family member) (61).

The Western blots in Fig. 6B reveal that the expression of 204AS (i.e. 204 antisense RNA) decreased the level of endogenous p204 in transfected NB508 cells (top). This decrease in turn was correlated with an increase in the phosphorylation of two mediators of Ras signaling, MEK and Akt, but with no change in the levels of total MEK and total Akt proteins (bottom four panels of Western blots). The extents of the decrease in p204 level and of the increase in phospho-MEK and phospho-Akt levels are shown in the diagrams on the right. Thus, the results in Fig. 6 revealed that an increase in p204 level inhibits, whereas a decrease in p204 level enhances, the phosphorylation (i.e. the activation) of Ras signaling by at least two mediators.

p204 Inhibited the Anchorage-independent Growth of Ras-transformed Cells—The inhibition of various Ras signaling pathways by p204 prompted us to explore the effects of p204 on the proliferation of H-rasQ61L-transformed cells in different growth conditions. The experiments involved the use of AKR-2B control cells (Con), AKR-2B Ind-p204 cells in which the expression of p204 was inducible by ponasterone A (Ind-p204), and derivatives of this cell line expressing ectopic H-RasQ61L, (H-RasQ61L-Ind-p204) (Fig. 7A).

Fig. 7B shows that the induction of p204 by ponasterone A resulted in an ~27% decrease in the rate of proliferation in monolayer culture of Ind-p204 cells and also of the H-RasQ61L-Ind-p204 cells. Thus, the extent of inhibition by p204 of the proliferation in monolayer cultures was not significantly affected in our conditions by the expression of ectopic H-RasQ61L.

When grown in suspension culture, the expression of H-RasQ61L oncprotein greatly increased colony formation (Fig. 7C, compare lower panels 1 and 3) (57, 62). In the absence of H-RasQ61L, only microcolonies were formed, whereas, in its presence, numerous colonies with a diameter over 1.5 μm were formed. This was expected, since, for proliferation, most types of untransformed cells require “anchorage,” (i.e. the attachment of their cell surface integrin receptors to the extracellular matrix). This attachment was found to generate signals that allowed anchorage-independent cell proliferation.

The induction of p204 strongly (by ~66%) inhibited the anchorage-independent growth of the H-RasQ61L-Ind-p204 cells (Fig. 7C, compare panels 3 and 4; see also Fig. 7D). This inhibition is in accord with the inhibition by p204 of the signaling triggered by RasGTP (Fig. 6).

Ras Oncoprotein Promoted a PI3K-dependent Translocation of p204 from the Nucleus to the Cytoplasm—The identity of a nuclear localization signal (NLS) (63) in p204 was established in experiments (Fig. 8) using monkey COS-7 cells expressing ectopic wild type or mutated murine p204 proteins. Sequences with 3 or more basic residues that might serve as an NLS in p204 are shown in boldface type in Fig. 8A. The arginine and lysine residues in three of these sequences were individually replaced by glutamine residues, and the effects of the replacements on the subcellular location of p204 are shown in Fig. 8, B, C, and D, right. The results established that the NLS determining the nuclear localization is the KKSAAK sequence extending in p204 from amino acid 188 to 194 (Fig. 8D).

The translocation of p204 from the nucleus to the cytoplasm during the differentiation of various cell types (12, 14) and the finding that p204 can modulate the signaling by the cytoplasmic Ras protein (this study) prompted us to study whether the expression of activated Ras can affect the subcellular location of p204.

For the study, we used three cell lines derived from MEF3T3. The removal of doxycycline (Tet) from the medium induced (i) in the MEF3T3-Tet-off-p204 line, the expression of p204, (ii) in the MEF3T3-Tet-off-RasG12V line, the expression of activated H-Ras, and (iii) in the MEF3T3-Tet-off-RasG12V-p204 line, the expression of both activated H-Ras and p204 (Fig. 9A).

The selective induction of p204 (in MEF3T3-Tet-off-p204) resulted in its expression in the nucleus (Fig. 9B); the induction of both p204 and activated H-Ras (in MEF3T3-Tet-off-RasG12V-p204) resulted in p204 distributed between the nucleus and the cytoplasm (Fig. 9C, top). The induction of H-Ras and p204 (in MEF3T3-Tet-off- RasG12V-p204), in the presence of a selective inhibitor of the PI3K pathway (LY294002) (64), however, resulted in the exclusively nuclear localization of p204 (middle). A different inhibitor (PD98059), blocking the signaling by the Raf1-MEK-ERK signaling pathway, had no effect on the distribution of p204 between the nucleus and the cytoplasm when induced together with Ras in the MEF3T3-Tet-off-RasG12V-p204 line (bottom).

These results indicate that activated Ras affected the subcellular distribution of p204 by triggering the appearance of a portion of p204 in the cytoplasm. Moreover, this effect of activated Ras required signaling by the PI3K pathway.

To generate more quantitative data on the effect of H-RasG12D induction on the distribution of p204 among different subcellular locations than obtained by visualization based on immunofluorescence (Fig. 9), we used Western blotting (Fig. S4). We fractionated cell lysates from MEF3T3-Tet-off-RasG12V cultures transfected with pCMV204 without, or after, inducing RasG12V expression (by removal of doxycycline) into nuclear, cytoplasmic, and crude membrane fractions and performed Western blotting using anti-p204. The results in Fig. S4 confirm and extend the conclusions in Fig. 9. They reveal that (i) prior to induction of RasG12V, the bulk of p204 is in the nuclear fraction, its presence in the cytoplasmic fraction is barely detectable, and none is detected in the crude membrane fraction; (ii) after induction of RasG12V, the majority of p204 is in the cytoplasmic fraction, and a lesser but significant amount of p204 is in the crude membrane fraction.
In the case of the translocation of p204 from the nucleus to the cytoplasm in the course of skeletal muscle myoblast and cardiac myocyte differentiation, the translocation was accompanied (possibly triggered) by the phosphorylation of p204 (12, 15). As shown in Fig. S5, the translocation elicited by Ras also appears to be accompanied by post-translational modification, including phosphorylation of p204. We did not attempt to identify the amino acid residue(s) phosphorylated in p204 in the course of the activated Ras-mediated translocation. We established, however (in experiments involving the expression of ectopic, wild-type, and mutant p204), that the replacement of serine at position 179 of p204 by alanine prevented the translocation from the nucleus to the cytoplasm, as triggered by activated Ras (Fig. 9D). The sequences at the 5' and 3' sides of serine 179 in p204 indicate that it is a potential target for phosphorylation by several kinases, including Akt (which is activated by PI3K, whose activity, as noted, is a prerequisite for the translocation) as well as calcium/calmodulin-dependent protein kinase (CaMK) and protein kinase A (65).

**Induction of Ras Oncoproteins in Cells Promoted an Alteration of the Actin Cytoskeleton and an Increase in the Rate of Cell Motility; Coinduction with p204 Inhibited Both of These Effects**—The effects of Ras oncprotein on the actin cytoskeleton and cell motility in the absence or presence of p204 were tested using cell lines in which removal of doxycycline (designated here as Tet) (i) induced H-Ras
tt^{G12V} (MEF3T3-Tet-off-H-Ras
tt^{G12V} line), (ii) induced p204 (MEF3T3-Tet-off-p204 line), and (iii) induced both H-Ras
tt^{G12V} and p204 (MEF3T3-Tet-off-H-Ras
tt^{G12V}-p204 line) (see Fig. 9A).

The MEF3T3-Tet-off cultures specified in Fig. 10 were stained with phalloidin to reveal their actin cytoskeleton and with DAPI to show their nuclei. The induction of H-Ras
tt^{G12V} resulted in (i) the alter-
Ras and various components of its signaling pathways were reported to promote cell motility (70–72). Fig. 10C (a and b) demonstrates a cell motility test based on the assaying of the number of cells passing through a membrane. It revealed that the induction of the H-RasG12V oncoprotein (in MEF3T3-Tet-off-H-RasG12V cells) increased the number of transmembrane cells ~5-fold (cf. a and b; see also the bottom diagram). The simultaneous induction of p204 with H-RasG12V decreased by ~70% the H-RasG12V-promoted increase in the number of transmembrane cells (cf. b and d; see also the bottom diagram). This inhibition by p204 of the H-RasG12V-promoted cell motility is also in accord with the inhibition by p204 of H-Ras signaling (Figs. 5 and 6).

The Egr-1 Transcription Factor (Whose Expression Is Known To Be Promoted by Activated Ras) Promoted the Expression of p204—Ras oncoprotein or wild-type Ras protein, when activated in cells exposed to growth factors, was reported to

FIGURE 8. Identification of an NLS in ectopic p204 protein in transfected monkey COS-7 cells. Left, sequences with three or more basic residues that are potential NLS-s are shown in boldface letters, above the schematic diagrams of p204 protein in A–D. The NES and the NLS identified are shown. The mutated amino acid residues (in which Lys residues were substituted by Gln residues) are printed in gray letters. (The significance of the KRKSMR sequence for subcellular localization is examined in Fig. 9D). Right, subcellular localization of wild type p204 (A) and of the mutated p204 protein with the amino acid substitutions indicated in the left panels (B–D). Shown is staining with antibodies to p204 and Tex-conjugated IgG (red) and of nuclei with DAPI (blue). For further details, see “Experimental Procedures.”

FIGURE 7. Induction of endogenous p204 inhibits the Ras oncoprotein-dependent, anchorage-independent proliferation of AKR-2B cells expressing ectopic H-RasG12V oncoprotein. A, the H-RasG12V-Ind-p204 cell line was constructed by transfection of a plasmid encoding H-RasG12V into AKR-2B Ind-p204 cells in which p204 expression is induced by ponasterone A (Ind-p204). A control line was constructed by transfection of the vector into AKR-2B cells (Con). Shown are assays of the levels of constitutive H-Ras expressions by cells of the Con line, the Ind-p204 line, and the H-RasG12V-Ind-p204 line by Western blotting with antibodies to H-Ras. As an internal control, β-tubulin was used. B, comparison of the growth in monolayer cultures of cells from the Con line, the Ind-p204 line, and the RasG12V-Ind-p204 line in the absence (gray) and the presence of ponasterone A (black). Equal numbers of cells were plated on day 0, and the cell numbers were determined after a 3-day incubation. C, induced endogenous p204 inhibits the anchorage-independent proliferation of AKR-2B cells expressing ectopic, activated RasG12V protein. Cells from the Ind-p204 line (1 and 2) and from the H-RasG12V-Ind-p204 line (3 and 4) were plated in soft agar in triplicates and were incubated without (1, 3) or with ponasterone A (2 and 4) for 2 weeks. The images were taken by a digital camera (top panels 1–4). The central areas of each panel were magnified 36-fold (bottom panels 1–4). The numbers in the top and bottom panels correspond to the same culture. D, diagram showing the inhibition of the H-RasG12V-dependent, anchorage-independent proliferation of AKR-2B cells by induced p204 (in 3 and 4). S.D. values are indicated. For further details, see “Experimental Procedures.”
increase the expression of the Egr-1 transcription factor (42, 73). We noted in the Ifi204 gene a binding sequence for Egr-1 (designated as S2 in Fig. 11A, a). A chromatin immunoprecipitation assay with anti-Egr-1 antibodies (but not preimmune IgG) revealed the coprecipitation of a chromatin segment from Ifi204 that was shown by PCR to include the region with the Egr-1 binding S2 sequence (Fig. 11A).

The transfection of an Egr-1 expression plasmid (pCMV-Egr-1) into a 3T3 culture (74) resulted in a strong increase in the level of endogenous p204 protein (Fig. 11A, d, top Western blot). The expression of a reporter construct (pGL-Egr-1) driving Luciferase and including the Egr-1-binding S2 sequence from the Ifi204 gene (but not that of a control reporter with a mutated Egr-1 binding sequence, pGLm-Egr-1) was strongly increased by the transfection of pCMV-Egr-1 into the 3T3 culture (Fig. 11A, d, right and left diagrams).

The presence of a functional Egr-1 binding sequence in the Ifi204 gene, whose expression was increased by ectopic Egr-1, together with the report (42) that activated H-Ras can increase the expression of Egr-1, prompted us to test whether the introduction into the 3T3 culture of activated H-Ras increases the level of endogenous p204. The data in Fig. 11B revealed that it did. Transfection into the 3T3 culture of the pCGN-H-RasQ61L expression plasmid (but not of the pCGN vector) increased the level of p204 (as well as of H-Ras) proteins (Fig. 11B, Western blot and diagram).

The transient activation of wild-type Ras (i.e. the conversion of Ras-GDP to RasGTP) in a serum-starved 3T3 culture by PDGF (Fig. 11C, top left, GST-Raf-1-RBD pull down assay followed by Western blotting with anti-H-Ras) resulted in an increase first in the level of Egr-1 protein and, after a delay, of p204 protein (as well as of H-Ras) proteins (Fig. 11B, Western blot and diagram).

The transient activation of wild-type Ras (i.e. the conversion of Ras-GDP to RasGTP) in a serum-starved 3T3 culture by PDGF (Fig. 11C, top left, GST-Raf-1-RBD pull down assay followed by Western blotting with anti-H-Ras) resulted in an increase first in the level of Egr-1 protein and, after a delay, of p204 protein (as well as of H-Ras) proteins (Fig. 11B, Western blot and diagram).
All of the above observations are in accord with the following interpretation: the increase in the level of activated Ras protein triggered (e.g. by PDGF) resulted in an increase in the level of Egr-1 transcription factor, which, in turn, increased the level of p204 expression. It is possible that besides Egr-1 also further transcription factors or other agents may be involved in mediating the increase in p204 level promoted by activated Ras. The above findings prompted us to test whether the induction of endogenous p204 (by removing doxycycline from the medium of a culture of MEF3T3-Tet-off p204 cells) affects the level of H-Ras. The data in Fig. 11D reveal no such effect. It is consistent with this result that p204 (which was shown to promote the proteasome degradation of Id proteins in consequence of promoting their ubiquitination (15)) did not promote the degradation of H-Ras (not shown).

Physiological Levels of Activated K-RasG12D Induced the Expression of p204 and Its Partial Translocation from the Nucleus to the Cytoplasm—The data in Fig. 11B revealed that expression of ectopic H-RasQ61D (in cells transfected with an H-RasQ61D expression plasmid) induced p204 expression. We wished to establish whether a physiological level of Ras oncoprotein present in cells carrying only a single copy of the ras oncogene would also result in an increased p204 level. For these studies, N. Bardeesy kindly provided MEF cultures. The MEFs were prepared from mice with the latent LSL-K-rasG12D allele (43) and from littermate controls. The LSL-K-rasG12D allele is not normally expressed due to the presence of a floxed stop cassette but can be activated by expression of Cre recombinase in these cells. Wild type control cells (11.1) infected with a Cre-GFP retrovirus (11.1wt/H11001 Cre) and LSL-K-rasG12D cells without Cre (11.7-Cre) expressed comparably low p204 levels, and exposure to 1000 units/ml interferon strongly increased this level. In contrast, LSL-K-rasG12D cells induced to express endogenous levels of K-RasG12D (11.7+C) (by infection with a Cre-GFP retrovirus, resulting in the deletion of the floxed stop cassette) exhibited greatly increased p204 levels; exposure of the culture to 1000 units/ml interferon increased this level only slightly (Fig. 12, six bottom panels). A quantitative comparison with RasG12V in MEF3T3-Tet-off-H-RasG12V, p204 cells inhibited both the rearrangement of the actin filament pattern and the acceleration of cell migration through the membrane. A, MEF3T3-Tet-off-H-RasG12V and MEF3T3-Tet-off-p204 cultures were incubated with doxycycline for 48 h to repress the expression of ras or p204, respectively (left), or without doxycycline to induce the expression of RasG12V or p204, respectively (right) for 48 h. The levels of Ras and p204 after the incubation are shown in Fig. 9A. The cultures were fixed with 3.7% formaldehyde. The actin filaments were stained with Alexa Fluor 555 phalloidin, and the nuclei were stained with DAPI. B, MEF3T3-Tet-off-H-RasG12V-p204 cell cultures were incubated without doxycycline to induce the expression of the H-RasG12V and p204 proteins. p204 was visualized by anti-p204 antibodies (top left) and actin by Alexa Fluor 555 phalloidin (top right). The actin and p204 staining were merged (bottom left), and so were staining with Alexa Fluor 555 phalloidin and DAPI (bottom right). C, MEF3T3-Tet-off-H-RasG12V cells (a and b) and MEF3T3-Tet-off-H-RasG12V-p204 cells (c and d) were seeded on transparent polyethylene terephthalate membranes (BD Company) with a pore size of 3.0 μm, in the presence of doxycycline (no induction (a and c)) or in the absence of doxycycline (induction of RasG12V (b) or of RasG12V and p204 (d). After a 5-day incubation, those cells that migrated to the other side of the membrane were stained with DAPI and photographed. The experiment was performed using cultures in triplicates. The diagrams in the bottom panel show the numbers of cells stained with DAPI that migrated through the membrane. a–d in the diagrams refer to the a–d panels above. The S.D. values are indicated. For further details, see “Experimental Procedures.”

FIGURE 10. Induction of H-RasG12V in MEF3T3-Tet-off-H-RasG12V cells resulted in the rearrangement of the actin filament pattern and the acceleration of cell migration through a membrane. Coinduction of p204
of the p204 levels in the cultures are shown in the Western blots and the diagram of Fig. 12. These revealed an ~5.5-fold increase in the p204 level in consequence of the expression of the single copy of the K-ras\textsuperscript{G12D} oncogene. This increase somewhat exceeded that triggered by 1000 units/ml interferon in the 11.1wt+Cre and the 11.7-Cre cultures. These results established that the presence of a single copy of the K-ras\textsuperscript{G12D} oncogene in a cell was sufficient to strongly boost p204 expression.

DISCUSSION

p204 was first found in the nucleolar and nucleoplasmic compartments of various cultured cells (75). In the nucleolus, it inhibits ribosomal RNA synthesis by blocking the activity of the UBF1 ribosomal RNA transcription factor (19). Its effects in the nucleoplasm include, for example, the enhancement of transcription by the Cbfa1 transcription factor by forming a ternary complex with pRb and Cbfa1 (17). During the differentiation of cultured myoblasts to myotubes and of cultured P19 embryonal carcinoma stem cells to cardiac-type myocytes, part of p204 is translocated from the nucleus to the cytoplasm (12, 14). Moreover, the bulk of p204 is cytoplasmic in isolated cardiac myocytes (12) and in heart slices from mice (Fig. 1B). A yeast two-hybrid assay (not shown) identified Ras, among other cytoplasmic proteins, that binds to p204. Pull-down assays (Fig. 2E) established that the binding between purified p204 and purified Ras was direct. The binding of endogenous p204 to endogenous Ras in mouse heart extract (Fig. 2A) and two murine tumor cell lines (Fig. 2C) revealed the physiological relevance of the binding. p204 bound both H-Ras (Fig. 2, A and B) and K-Ras (Fig. 2, C and D).

The fact that p204 bound to H-RasGTP (but not to H-RasGDP) (Fig. 2F) supported the assumption that, similarly to RasGAP and various Ras effectors, p204 might also bind to the Ras effector domain (although not necessarily exclusively). The validity of this assumption was strengthened by the finding that p204 inhibited the activity of RasGAP (Figs. 4B and S3) and also inhibited the binding to H-Ras of the Ras effectors (i) Raf-1 (actually, Raf-1 RBD was tested, since Raf-1 also binds a second domain in H-Ras) (76), (ii) PI3K, and (iii) RalGDS (Fig. 5, A–C).

Induction of p204 also inhibited signaling (as assayed by testing for target phosphorylation) by some Ras effectors including (i) phosphorylation of MEK promoted by Raf-1, (ii) phospho-
rylation of Akt promoted indirectly by PI3K, and (iii) phosphorylation of p38MAPK as promoted directly or indirectly by several of the Ras signaling proteins Tiam, Rac, PI3K, and MKK3/6 (Fig. 6A). A decrease in the level of endogenous p204, however, resulted in an increase of the phosphorylation of MEK and Akt (Fig. 6B). In addition to Raf-1, PI3K, and RafGDS, several further Ras effectors were identified (35, 36). It remains to be established which of these are prone to inhibition by p204.

A comparison of the binding to H-RasGTP of p204 and its various segments revealed that both p204 and its N segment bind to H-RasGTP. The N segment includes among other regions two characteristic domains: (i) an ~95-amino acid-long sequence of the PAAD/DAPIN/Pyrin domain type (77) (such domains occur at the N termini of several proteins involved in apoptosis and inflammatory signaling pathways) and (ii) a segment consisting of eight imperfect repeats of a 7-amino acid sequence (7). It remains to be seen whether (i) or (ii) are involved in the binding of p204 to RasGTP.

The induction of p204 inhibited the anchorage-independent growth of H-RasQ61L-expressing cells in suspension culture involved in the binding of p204 to RasGTP. The N segment includes among other regions two characteristic domains: (i) an ~95-amino acid-long sequence of the PAAD/DAPIN/Pyrin domain type (77) (such domains occur at the N termini of several proteins involved in apoptosis and inflammatory signaling pathways) and (ii) a segment consisting of eight imperfect repeats of a 7-amino acid sequence (7). It remains to be seen whether (i) or (ii) are involved in the binding of p204 to RasGTP.

The induction of p204 inhibited the anchorage-independent growth of H-RasQ61L-expressing cells in suspension culture much more strongly than their growth in monolayer culture (Fig. 7, B–D). This is in accord with (i) the strong dependence of anchorage-independent growth (but not growth in monolayer culture) on the constitutive expression of Ras oncproteins (57, 62) and (ii) the inhibition of Ras signaling by p204 (Figs. 5 and 6).

The condition-dependent nuclear localization of p204 in various cell types prompted us to identify its NLS. This was found to be the KKSKAAG sequence extending from amino acid 188 to 194 in the p204 protein (Fig. 8). This sequence fits the conclusions of a structural study concerning the key requirements for a monopartite NLS; it contains Lys residues in its positions 1, 2, and 4 (of these, Lys in positions 2 and 4 could be substituted by Arg) (78, 79). We did not rule out the possibility, however, that the KRRS sequence extending from amino acid 176 to 179 in p204 may be part of a bipartite NLS sequence whose second part might be the above discussed KKSKAAG sequence.

The induction of constitutively active Ras oncprotein in MEF-3T3-Tet-off-RasG12V cells resulted in the translocation of the bulk of p204 from the nucleus to the cytoplasm (Fig. 9C). A more quantitative demonstration of the redistribution of p204 from the nuclear to the cytoplasmic and crude membrane fractions is shown in the Western blots of Fig. S4.) The translocation of p204 from the nucleus did not take place (i) in the presence of an inhibitor of PI3K (Fig. 9C) or (ii) if Ser179 in p204 was replaced by Ala (Fig. 9D). As noted earlier, the Ser179 is part of a KRKKSR sequence. It is a potential target of phosphorylation by cyclic AMP-activated protein kinase, by CaMK, and by Akt (that is activated by PI3K) (65). p204 contains several additional sequences that are potential targets of the above kinases.

It may be relevant that the deletion from histone deacetylase 5 of two consensus sequences of the above type that are targets of phosphorylation by, among others, CaMK, inhibited the CaMK-mediated nuclear export of histone deacetylase 5 (80).

The data in Fig. S5 reveal that expression of ectopic H-RasG12V triggered the phosphorylation of p204. We have made no attempt to identify the site(s) phosphorylated in p204, the kinase(s) responsible, or other posttranslational modifications (if any) triggered in p204 by activated Ras. We established, however, that the induction of the catalytic subunit of cyclic AMP-activated protein kinase in NIH3T3 cells or the transfection of CaMK into cultured C2C12 myoblasts resulted in the translocation of p204 from the nucleus to the cytoplasm (data not shown).

This translocation of p204 occurs also in the course of the differentiation of C2C12 myoblasts (12) to myotubes and of P19 embryonal carcinoma stem cells to beating cardiac-type myocytes (14). p204 contains a classical NES (whose position is indicated in Fig. 8) known to be required for the translocation of p204 from the nucleus to the cytoplasm. Ectopic (wild-type) p204 promoted the differentiation of P19 cells to cardiac-type myocytes, whereas p204 from which the NES was deleted or 3 of the Leu residues in NES were replaced by Ala residues did not promote it (14). Moreover, p204 isolated from undifferentiated myoblasts was unphosphorylated, whereas p204 from the cytoplasmic fraction (but not from the nuclear fraction) of differentiated myotubes was phosphorylated (12). p204 was also found to promote the translocation from the nucleus to the cytoplasm of Id proteins (15). p204 binds the Id proteins and accelerates their degradation by promoting their ubiquitination (15). It is conceivable that the inhibition of Ras activity by p204 in the
ERK1/2; Ras signaling via the PI3K-phosphatidylinositol-triphosphate pathway promotes the activation, among others, of RhôGEF; and Ras signaling via the RalGEF pathway promotes the activation of the Rac and Cdc2 proteins (70, 72).

The ERK1/2, Rho, Rac, and Cdc2 family GTPases, among others, in turn, promote changes in the actin cytoskeleton and in the adhesion to the extracellular matrix (62, 71). They also elicit extensions of fingerlike filopodia and broad lamellopodia-type ruffles from the plasma membrane (66, 67). All of the above effects of signaling by Ras contribute to the alterations of cell morphology (cf. Fig. 9A, a and b).

Simultaneous induction of p204 with H-RasG12V inhibited the above changes (Fig. 10B). This inhibitory effect of p204 is consistent with its inhibition of signaling by Ras via several pathways (Figs. 5 and 6).

Ras oncprotein is known to enhance cell motility, which promotes, among other activities, the invasiveness and metastatic activity of tumor cells (70, 71). Cell motility requires constant changes in the cell morphology (i.e. constant restructuring of the actin cytoskeleton in different parts of the cell, together with constant making and breaking of the attachments of different portions of the cell to the extracellular membrane). Consequently, the same proteins that are activated by Ras signaling pathways and modulate the cell morphology are also involved in enhancing cell motility.

Oncogenic RasG12V increased the motility of MEF3T3 cells, as determined in a transmembrane migration assay, ~5-fold, and the induction of p204 diminished this increase by ~70% (Fig. 10C). This effect of p204 was in accord with its inhibition of Ras signaling.

The finding of a binding sequence for the Egr-1 transcription factor (81) in the Ifi204 gene prompted us to verify (i) the activity of this sequence when inserted into a reporter construct driven by Egr-1 and (ii) the induction of endogenous p204 by ectopic Egr-1. The verification of (i) and (ii) (Fig. 11A (d)) and the fact that Egr-1 expression can be activated by Ras oncprotein and also by PDGF (primarily via the Ras-Raf-MEK-ERK pathway) (42, 73, 82) prompted us to test whether activated RasQ61L or PDGF does induce the expression of p204. The results in Fig. 10, B and C, established that both did. An increase in the level of p202 (7), a sister protein of p204, was reported in H-ras oncogene-transformed NIH3T3 cells (83).

Egr-1 is known to induce the expression of its corepressor Nab2 (84). This induction can be accounted for by the presence of a series of Egr-1 binding sequences in the promoter of the Nab2 gene. Nab2 protein expression follows that of Egr-1 by several h. Nab2 binds and inhibits the activity of Egr-1, thus making the expression of the Egr-1 target genes transient.

The findings that (i) activated Ras promoted the expression of p204 via the Ras-Egr-1-p204 pathway and (ii) p204 inhibited Ras signaling (this study) suggest that p204 might be considered as one of the negative feedback inhibitors of Ras. It should be noted that the induction of p204 had little or no effect on the level of H-Ras (Fig. 11D).

Egr-1 plays a master regulatory role in multiple cardiovascular pathological processes (85). It remains to be established whether Egr-1 activity contributes to the high level of p204 in heart tissue.
The triggering by the ras oncogene of (i) the translocation of p204 from the nucleus to the cytoplasm (Fig. 9) and (ii) the induction of p204 expression (Fig. 11) were tested by using induced (Fig. 9) or ectopic ras oncogene (Fig. 11). To extend the relevance of these observations to a case analogous to that in which a single copy of the ras oncogene arises in a cell (in vivo), we performed the following experiments (Fig. 12). In these, the effect of the removal (by Cre) of a transcriptional stopper from a single copy of K-ras oncogene in murine embryonic cells was tested on p204 expression and subcellular localization. This removal (which allowed the expression of the oncogene) triggered the expression of p204 at a level similar to that of the exposure of the culture to 1000 units/ml α-interferon as well as the redistribution of p204 between the nucleus and the cytoplasm.

These results suggest that “physiological” levels of ras oncogene can trigger the expression of high levels of p204, and thus, the mutational activation in vivo of a single copy of a ras gene may generate enough p204 to serve as its negative feedback inhibitor. Thus, it will be of interest to explore the effects of the cell-specific conditional inactivation in vivo of the gene encoding p204 on the tumorigenic activity of cells with a single copy of the ras oncogene.

A pioneering study, using MEFs, in which only a single copy of the K-rasG12D oncogene was expressed, unexpectedly revealed an attenuation of Ras signaling (compared with the signaling in wild-type cells) via the Raf and PI3K pathways (43). However, the protein levels of Raf, MEK, Erk, PI3K, and Akt were unaffected by the presence of the ras oncogene. No mechanism for the attenuation of Ras signaling was proposed.

The results presented in this study may provide such a mechanism (Fig. 13). This can be based on the findings that (i) activated wild-type or oncogenic Ras did induce p204 synthesis (mediated by Egr-1, whose expression was increased in consequence of Ras signaling) (42, 73) (Fig. 11), (ii) a single copy of K-rasG12D in a cell induced a high level of p204 (Fig. 12), (iii) p204 bound RasGTP and inhibited Ras signaling via the Raf and PI3K (and other) pathways. Decreasing the level of endogenous p204 in cells expressing a single copy of K-rasG12D by ectopic 204 antisense RNA, however, resulted in the increase in Ras signaling via the Raf and PI3K pathways (Fig. 6B). It is conceivable that, besides p204, other agents might be induced by Ras that also cause or contribute to the attenuation of Ras signaling.

Types of proteins were described that, similarly to p204, bind Ras proteins and (unlike Ras effectors) inhibit Ras signaling (86). Carabin (an endogenous inhibitor of the phosphatase activity of calcineurin in T cells (87)) exhibits RasGAP activity. Members of the Ras association domain family (45, 88) can interact directly with RasGTP. The biological characteristics of Ras association domain family proteins suggest that they are tumor suppressors. Sprouty (Spry) proteins function as suppressors of K-Ras-mediated tumorigenesis (89). There are no published data on a direct interaction between Ras and Spry proteins.

The recently described human Sin1 (stress-activated protein kinase-interacting protein) contains a Ras binding domain, it binds activated H- and K-Ras protein in vivo and in vitro, and its overexpression inhibits the activation of the ERK, Akt, and Jun terminal kinase signaling pathways (90). Thus, among the Ras-binding proteins described, the Ras inhibitory activity of the murine p204 protein resembles that of the human Sin1 protein the most.

The results of this study established that activated Ras can induce the synthesis of p204 and, by binding to RasGTP p204, can inhibit the signaling by activated Ras. It is conceivable that, in addition to inhibiting the signaling by numerous Ras effectors, p204 might also initiate as yet unidentified signals if triggered by binding RasGTP.

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REFERENCES
1. Sen, G. C. (2001) Annu. Rev. Microbiol. 55, 255–281
2. Sarkar, S. N., and Sen, G. C. (2004) Pharmacol. Ther. 103, 245–259
3. Samuel, C. E. (2007) J. Biol. Chem. 282, 20045–20046
4. Deschamps, S., Meyer, J., Chatterjee, G., Wang, H., Lengyel, P., and Roe, B. A. (2003) Genomics 82, 34–46
5. Ludlow, L. E., Johnstone, R. W., and Clarke, C. J. (2005) Exp. Cell Res. 308, 1–17
6. Ding, Y., Lee, J. F., Lu, H., Lee, M. H., and Yan, D. H. (2006) Mol. Cell Biol. 26, 1979–1996
7. Choubey, D., Snoddy, J., Chaturvedi, V., Tonieto, E., Opdenakker, G., Thakur, A., Samanta, H., Engel, D. A., and Lengyel, P. (1989) J. Biol. Chem. 264, 17182–17189
8. Min, W., Ghosh, S., and Lengyel, P. (1996) Mol. Cell. Biol. 16, 359–368
9. Choubey, D., and Kotzin, B. L. (2002) Front. Biosci. 7, e252–262
10. Ma, X. Y., Wang, H., Ding, B., Zhong, H., Ghosh, S., and Lengyel, P. (2003) J. Biol. Chem. 278, 23008–23019
11. Asefa, B., Dermott, J. M., Kaldis, P., Stefanisko, K., Garfinkel, D. J., and Keller, J. R. (2006) FEBS Lett. 580, 1205–1214
12. Liu, C., Wang, H., Zhao, Z., Yu, S., Lu, Y. B., Meyer, J., Chatterjee, G., Deschamps, S., Roe, B. A., and Lengyel, P. (2000) Mol. Cell. Biol. 20, 7024–7036
13. Liu, C. J., Ding, B., Wang, H., and Lengyel, P. (2002) Mol. Cell. Biol. 22, 2893–2905
14. Ding, B., Liu, C. J., Huang, Y., Hickey, R. P., Yu, J., Kong, W., and Lengyel, P. (2006) J. Biol. Chem. 281, 14882–14892
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Ding, B., Liu, C. J., Huang, Y., Yu, J., Kong, W., and Lengyel, P. (2006) J. Biol. Chem. 281, 14893–14906

Liu, C. J., Chang, E., Yu, J., Carlson, C. S., Pazarak, L., Yu, X. P., Ding, B., Lengyel, P., and Di Cesare, P. E. (2005) J. Biol. Chem. 280, 2788–2796

Luan, Y., Yu, X. P., Xu, K., Ding, B., Yu, J., Huang, Y., Yang, N., Lengyel, P., Di Cesare, P. E., and Liu, C. J. (2007) J. Biol. Chem. 282, 16860–16870

Dauffy, J., Mouchiroud, G., and Bourrette, R. P. (2006) J. Leukocyte Biol. 79, 173–183

Liu, C. J., Wang, H., and Lengyel, P. (1999) EMBO J. 18, 2845–2854

Hertel, L., Rolle, S., De Andrea, M., Azzimonti, B., Osello, R., Gribaudo, G., Gariglio, M., and Landolfo, S. (2000) Oncogene 19, 3598–3608

Rolle, S., De Andrea, M., Gioia, D., Lembo, D., Hertel, L., Landolfo, S., and Gariglio, M. (2001) Virology 286, 249–255

Shah, C., and Weinberg, R. A. (1982) Cell 29, 161–169

Goldfarb, M., Shimiziu, K., Perucchi, M., and Wigler, M. (1982) Nature 296, 404–409

Pulciani, S., Santos, E., Lauver, A. V., Long, L. K., Robbins, K. C., and Barbacid, M. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2845–2849

Malumbres, M., and Barbacid, M. (2003) Nat. Rev. Cancer 3, 459–465

Weinberg, R. B. (2006) The Biology of Cancer. Garland Science

Boguski, M. S., and McCormick, F. (1993) Nature 366, 643–654

Willumsen, B. M., Christensen, A., Hubbert, N. L., Papageorge, A. G., and Lowy, D. R. (1984) Nature 310, 583–586

Jura, N., and Bar-Sagi, D. (2006) Cell Cycle 5, 2744–2747

Sheils, J. M., Pruitt, K., Mcfall, A., Shaub, A., and Der, C. J. (2000) Trends Cell Biol. 10, 147–154

Gibbs, J. B., Sigal, I. S., Poe, M., and Scolnick, E. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 5704–5708

McGrath, J. P., Capon, D. J., Goeddel, D. V., and Levinson, A. D. (1984) Nature 310, 646–649

Sweet, R. W., Yokoyama, S., Kamata, T., Feramisco, J. R., Rosenberg, M., and Gross, M. (1984) Science 224, 352–355

Rodriguez-Viciana, P., Warner, P. H., Khwaja, A., Marte, B. M., Pappin, D., Das, P., Waterfield, M. D., Ridley, A., and Downward, J. (1997) Cell 89, 457–467

Lee, S., and Helfman, D. M. (2004) J. Biol. Chem. 279, 1885–1891

Charest, P. G., and Firtel, R. A. (2007) Biochem. J. 401, 377–390

Oxford, G., and Theodorescu, D. (2003) Cancer Lett. 199, 117–128

Goldberg, L., and Kroog, Y. (2006) Cancer Res. 66, 11709–11717

Thiel, G., and Cibelli, G. (2002) J. Cell Physiol. 193, 287–292

Classon, M., Kennedy, B. K., Mulloy, R., and Harlow, E. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 10826–10831

Chouday, B., and Lengyel, P. (1992) J. Cell Biol. 116, 1333–1341

Shirouzu, M., Koide, H., Fujita-Yoshigaki, I., Oshio, H., Toyama, Y., Yasumaki, K., Fuhrman, S. A., Villafranca, E., Kaziro, Y., and Yokoyama, S. (1994) Oncogene 9, 2153–2157

Du, K., and Pio, F. (2006) FEBS Lett. 580, 3083–3090

Conti, F., and Kuriyan, J. (2000) Structure 8, 329–338

Fontes, M. R., Teh, T., and Kobe, B. (2000) J. Mol. Biol. 297, 1183–1194

McKinsey, T. A., Zhang, C. L., Lu, J., and Olson, E. N. (2000) Nature 408, 106–111

Gashler, A., and Sukhatme, V. P. (1995) Prog. Nucleic Acids Res. Mol. Biol. 50, 191–224

Hjoberg, J., Le, L., Imrich, A., Subramaniam, V., Mathew, S. L., Vallone, J., Haley, K. J., Green, F. H., Shore, S. A., and Silverman, E. S. (2004) An. J. Physiol. 286, 1817–1825

Xin, H., Geng, Y., Pramanik, R., and Choubey, D. (2003) J. Cell Biochem. 88, 191–204

Kumbrink, J., Gerlinger, M., and Johnson, J. P. (2005) J. Biol. Chem. 280, 42785–42793

Agathangelou, A., Cooper, W. N., and Latif, F. (2005) Cancer Res. 65, 3497–3508

Shaw, A. T., Meissner, A., Dowdle, J. A., Crowley, D., Magendanz, M., Ouyang, C., Parisi, T., Blank, L. J., Bronson, R. T., Stone, J. R., Tuveson, D. A., Jaenisch, R., and Jacks, T. (2007) Genes Dev. 21, 694–707

Schroder, W. A., Buck, M., Cloonan, N., Hancock, J. F., Suhre, A., Scully, T., and Bushell, G. (2007) Cell. Signal. 19, 1279–1289