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ARTICLES

Nuclear Ras: Unexpected Subcellular Distribution of Oncogenic Forms

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Abstract The Harvey-*ras* gene encodes small guanine nucleotide binding proteins, mutant forms of which are associated with a number of human malignancies. Based on studies with truncated forms of the protein it is known that correct post-translational processing of Ras is essential for cytoplasmic membrane localization and function. Surprisingly, immunofluorescence analysis provided evidence that in addition to its cytosolic localization, activated H-Ras$^{Val\ 12}$ was also localized in the nuclei of transformed cells both in vitro and in vivo. Immunoblot analysis of nuclear fractions was consistent with results found by immunohistochemistry. Moreover, inhibition of protein farnesylation prevented the nuclear targeting of activated H-Ras$^{Val\ 12}$ and NFκB. Alterations in subcellular distribution pattern and phosphorylation of the cell cycle inhibitor p27, which is involved in Ras driven tumor growth, coincided with nuclear localization of H-Ras$^{Val\ 12}$. Proteins are often not functional until they are transported to their final destination. Indeed, Ras was found to complex with NTF2 a factor involved in nuclear protein import and export. Therefore it is suggested that NTF2 is the actual carrier for oncogenic Ras. In view of these observations the question arises whether the nuclear localization of H-Ras$^{Val\ 12}$ in tumors is important in oncogenic activation or whether it is a response to apoptosis. J. Cell. Biochem. Suppl. 36:1–11, 2001.

Key words: H-Ras nuclear localization; Ras CAAX farnesylation; GTPase Ran and NTF2; NF-kappaB; hepatocarcinogenesis in mouse liver; endoplasmic reticulum; Golgi apparatus

The cellular Harvey Ras oncoprotein is a prominent representative of small G-proteins and a central point of convergence for a number of signaling pathways that cause alterations within cells. Regulators like GTPase-activating proteins (GAPs) [Boguski and McCormick, 1993; Macara et al., 1996] stimulate two intrinsic activities of Ras: 1) the hydrolysis of GTP and 2) GDP/GTP exchange. Moreover, mutations in codon 12, 13 or 61 give rise to forms of Ras that are constitutively active. Ras activity modulates the cell's fate in diametrically different directions including both apoptosis and cell proliferation. It is suggested that the final outcome in response to Ras is dictated by the relative activation levels of different effector pathways. A promising strategy for combating Ras-mediated transformation was prompted by the finding that functional Ras depends on posttranslational modifications [Kato et al., 1992]. In general, members of the Ras super-family of small GTPases undergo different types of posttranslational modifications at their C-terminal CAAX motives, prenylation, endoproteolytic cleavage and carboxymethylation. An important consequence of prenylation of the cytosolic precursor is membrane binding, thereby enabling Ras to act as a GTP-dependent, membrane localized docking site for proteins. Prevention of posttranslational modifications with specific inhibitors of farnesyltransferase causes mislocalization of Ras proteins within the cells [Omer and Kohl, 1997].

Nucleoplasmatic transport of proteins requires a precisely orchestrated series of interactions between pore components and soluble factors. Ran, another member of the

Abbreviations used: H-Ras, Harvey-Ras; NTF2, nuclear transfer factor 2; GTP, guanosine triphosphate; GDP, guanosine diphosphate; NFκB, nuclear factor kappa B; Cdk2, cyclin-dependent kinase-2; p27, p27^{Kip1}; NDEA, N-nitrosodiethylamine; ts p53, temperature-sensitive p53; FPTase β, Farnesyl Protein Transferase β.

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Ras-family of GTPases is suggested to be a key player in this process [Izaurralde et al., 1997; Richards et al., 1997]. Asymmetrical distribution between the cell compartments assigns different functions to the cytoplasma associated Ran-GDP and nucleic Ran-GTP [Nachury and Weis, 1999]. In contrast to nuclear import, it has been suggested that export requires the presence of the GTP-bound form of the Ras-related GTPase Ran and is stimulated among other proteins by nuclear transfer factor 2 (NTF2) [Englmeier et al., 1999].

Extending this avenue of research, the aim of this study was to focus on proteins, known to be engaged in Ras mediated transformation. Therefore, the expression pattern of the cyclin-dependent kinase-2 (Cdk2) cell cycle inhibitor $p27^{Kip1}$ (p27) was characterized in context with nuclear localization of oncogenic H-Ras. Repression of the p27 synthesis [Morisset et al., 1999], phosphorylation at Thr(187) [Tsvetkov et al., 1999] and Ser(10) [Ishida et al., 2000] and cytoplasmic sequestration [Reynisdottir and Massague, 1997] are different mechanisms regulating the stability of the cell cycle regulator.

A previous study on the distribution pattern of Ras revealed perinuclear expression of the protein in addition to plasma membrane staining and requests to reinterpretate subsequently published immunofluorescent studies [Choy et al., 1999]. Accordingly, this study delineates a novel subcellular distribution pattern of activated H-Ras proteins in vitro and in vivo providing evidence for nuclear localization of the oncoprotein. A better understanding of factors which influence Ras distribution pattern could be beneficial to design novel strategies aimed at inhibition of Ras mediated transformation. In order to address this question the present study has focused on proteins known to be involved in nuclear import and export.

MATERIAL AND METHODS

Materials

Antibodies used in the present study were from the following sources: anti-p21/c-H-Ras mouse monoclonal antibody—DAKO Corporation (Glostrup, Denmark); anti-p53 (Ab-1) mouse monoclonal antibody—Oncogene Research Products (Cambridge, MA); anti-p27 mouse monoclonal antibody—Santa Cruz Biotechnology (Santa Cruz, Ca); anti-NTF2 antibody—Transduction Laboratories (Lexington, KY); anti-lamin A/C mouse monoclonal antibody—(41CC hybridoma cell line) a kind gift of Dr. Brian Burke; anti-Farnesyl Protein Transferase β (FPTase β) - Transduction Laboratories (Lexington, KY); anti-NF kappaB (NFkB) p65 (C-20) rabbit polyclonal antibody—Santa Cruz Biotechnology (Santa Cruz, Ca); horseradish peroxidase (HRP)-conjugated secondary antibodies—DAKO Corporation (Glostrup, Denmark); fluorescein-conjugated anti mouse IgG antibody—Amersham International (Little Chalfont, Buckinghamshire, England); normal goat serum—DAKO Corporation (Glostrup, Denmark). Cell culture media, antibiotics and solutions were obtained from Sigma (Germany). Fetal calf serum (FCS) was purchased from Sigma (Germany) and FPTase inhibitor (AL-290005 Farnesyltransferase Inhibitor Cys-R-val-R-phe-m) was obtained from Alexis Biochemicals (Lausen, Switzerland). Molecular biology reagents were purchased from New England Biolabs (Beverly, Ma), Promega (Madison, WI), Amersham International (Little Chalfont, Buckinghamshire, England) and Boehringer Mannheim Biochemicals (Indianapolis, IN). Reagent grade chemicals were obtained from BioRad (Hercules, CA) and Sigma (Germany).

Animal Treatment

Male C3H/He were purchased from the Forschungsinstitut für Versuchstierzucht und -haltung (Himberg, Austria). All mice were obtained at 3 weeks of age and were housed individually in plastic cages on standard softwood bedding (Altromin, Lage, Germany) under standardized conditions. At 5 weeks of age, the mice received a single intraperitoneal injection of N-nitrosodiethylamine (Sigma Chemical., Germany; 90mg/kg of body weight) freshly dissolved in sterile 0.9% saline (20 mL/kg of body weight). The mice in control groups received 0.9% saline (20 mL/kg of body weight) only. From 7 weeks of age, NDEA-treated mice were kept on standard diet and were continuously fed a diet containing an adjusted concentration (0.05-0.07%) of Phenobarbital (Fluka Chemie, Buchs, Switzerland) yielding a dose of approximately 90 mg/kg of body weight. Body weights and food consumption were recorded at regular intervals. The mice were killed 52 weeks after NDEA or saline injection, i.e., at 57 weeks of age. Animals were weighed,

anaesthetized with carbon dioxide, decapitated, and exsanguinated. The liver was removed and examined for gross lesions. Lesions of 10–20 mm in diameter were excised and used for immunohistochemical analysis.

Cell Culture

Primary rat embryonic cells cotransformed with activated human H-Ras$^{Val\ 12}$ and ts p53$^{Val\ 135}$ or c-Myc were grown in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10% FCS in an atmosphere of 7.5% CO_2 and harvested with a rubber policeman. For total protein lysates, cells were washed three times with ice cold phosphate buffered saline (PBS) and proteins were extracted for 40 minutes with RIPA buffer (50 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS and 0.05% NaN_3).

Cell Treatment

For FPTase β inhibition studies, cells were plated on slides and grown for 24 hours in DMEM containing 10% FCS and 50 μM FPTase β inhibitor before fixation and labeling with anti-p21/c-H-Ras or anti-NFκB antibodies.

Immunocytochemistry of Cells

Cells were grown on coverslips, rinsed with PBS, fixed in ice-cold methanol-acetone (3:2) for 20 min, and permeabilized with 0.2% Triton X-100 in PBS for 15 min. After washing, cells were blocked with PBS containing 5% dry defatted milk. The cells were incubated with anti-p21/ras or anti-NFκB antibodies, both diluted 1:800 in 0.01% Tween-PBS containing 1% dry defatted milk for 1 hour, and washed five times for 10 min with 0.01% Tween-PBS. Immune complexes were detected by incubation with secondary antibodies covalently coupled to Cy-2 or Cy-3 (Amersham International; Little Chalfont, Buckinghamshire, England). For visualization of nuclei, cells were stained for 10 min with 4,6-diamidino-2-phenylindole (1 μg/ml) in Mounting Medium (DAKO Corporation; Glostrup, Denmark).

Electron Microscopy

Cultured cells were fixed in situ by incubation in 2% freshly depolymerized paraformaldehyde in PBS (pH 7.4) for 20 minutes, collected with a cell scrapper, and centrifuged in the fixative (400 x *g*) for 10 minutes. The fixative was discarded, the pellet resuspended in PBS and centrifuged again twice to remove unbound aldehydes. The cell pellet was then processed for electron microscopy analysis as described previously [Mosgoller et al., 1993]. For electron microscopy labeling of Ras and p53 was performed using monoclonal mouse anti-p21/ras antibodies (1:200) and monoclonal anti-p53 (Ab-1) immunoglobulines (1:100), both diluted in PBS containing 5% fetal calf serum. Immunodetection and section processing followed previously published methods using 5 nm colloidal gold as marker. For negative controls the primary antibody was substituted by FCS, which abolished labeling on the sections. Sections were examined in a Jeoul EM 1200 or in a Zeiss EM900 transmission electron microscope.

Immunohistochemistry of Liver

Serial liver sections (3–5 μm thick) from mice treated with NDEA and Phenobarbital were prepared from paraffin-embedded tissue as previously described [Wastl et al., 1998]. The sections were deparaffinized in xylene, endogenous peroxidase was inactivated by incubation in 2% hydrogen peroxide in methanol. Nonspecific binding sites were blocked with normal goat serum diluted 1:30 with PBS. The sections were then incubated with anti-p21/ras immunoglobulins diluted 1:200 in PBS, rinsed and incubated with a horseradish peroxidase coupled anti-mouse antibody, diluted 1:200 with PBS. Diaminobenzidine was applied as the chromogen and nuclei were counterstained with 1% hematoxylin.

Nuclear Extracts

PBS washed cells were suspended in low salt buffer (10 mM Tris/HCl pH 7.4, 10 mM NaCl, 1.5 mM $MgCl_2$) and swollen for 10 min at 4°C. Cells were centrifuged and resuspended in low salt buffer with sodium deoxycholate and Nonidet P-40 at final concentrations of 0.5% and 1%, respectively. Cells were homogenized in a Potter-Teflon/glass homogenizer and nuclei were sedimented through a cushion of 0.8 M sucrose, 10 mM Tris/HCl pH 7.4, 3 mM $MgCl_2$ to remove cytoplasma.

Immunoprecipitation

Nuclear fractions of normal RECs and RECs transformed with activated human H-Ras$^{Val\ 12}$ and complementing oncogenes temperature sensitive *p53$^{Val\ 135}$* or c-*myc* were incubated with the appropriate antibody (anti-p21/ras or

anti-NTF2) [Wesierska-Gadek et al., 1998] for one hour at room temperature. After addition of prewashed Gamma-Bind Sepharose beads (Pharmacia), the incubation was continued for two hours. The supernatant was discarded and beads were washed extensively. Isolated immune complexes were separated on 16% polyacrylamide gels (Novex). NTF 2 proteins in complexes immunoprecipitated from nuclear fractions with anti-H-Ras antibody were detected with anti-NTF2 antibodies.

Western Blotting

Extracted proteins of nuclear fractions and RIPA lysates were separated by 15% SDS-PAGE and transferred to polyvinylidene difluoride membranes (PVDF). Membranes were blocked by Phosphate-buffered saline containing 5% skim milk, probed with adequate dilutions of the primary antibody (H-Ras, Lamin A/C, FPTase β, NTF2, and p27), followed by horseradish peroxidase-conjugated secondary antibody as appropriate. Blots were incubated with a chemiluminescence substrate (ECL+ reagent; Amersham Life Science, Little Chalfont, UK) and exposed to X-OMAT/LS films (Eastman Kodak, Rochester, NY).

RESULTS

Subcellular Distribution of Activated H-Ras Oncoprotein

Very recently, enzymes modifying prenylated Ras proteins were found to be restricted to the endomembrane system of the Golgi apparatus and the endoplasmic reticulum [Choy et al., 1999]. The requirement of endomembrane targeting of immature Ras proteins trafficking from the cytosol to the plasma membrane shed new light on the intracellular localization of H-Ras$^{Val\ 12}$. To determine the distribution pattern of Ras proteins in rat embryonic cells transformed with activated human H-Ras$^{Val\ 12}$ and complementing ts p53$^{Val\ 135}$ or oncogenic c-Myc a monoclonal p21/ras antibody directed against the activated form of H-Ras was used for confocal (Fig. 1) and electron microscopy (Fig. 2A). Indeed, confocal microscopy revealed nuclear localization of oncogenic H-Ras$^{Val\ 12}$ in cells cotransformed with mutant p53 (Fig. 1). Moreover, electron microscopy extended the finding of nucleic H-Ras$^{Val\ 12}$ protein to c-Myc and activated H-Ras$^{Val\ 12}$ transformed cells. The cytoplasm and nucleoplasm of transformed cells was stained, and most of the Ras label was associated with the transition zone between condensed chromatin and electron translucent nucleoplasm (arrows). Within the nucleus most of the grains were at the periphery of condensed chromatin which is found close to the nuclear envelope or deeper within the nucleus. The bulk of condensed chromatin was devoid of label. Data obtained from electron microscopy of cells labeled with antibodies against nucleic wild type p53 compared well with subcellular distribution pattern of activated H-Ras (Fig. 2B). Occasionally, some p53 protein was found in the cytoplasm, but most of the signal is associated with condensed or decondensed chromatin within the nucleus. This observation substantiated the observation that activated H-Ras$^{Val\ 12}$ localizes in the nucleus of transformed cells. To further confirm the presence of nuclear H-Ras$^{Val\ 12}$, transformed cells were fractionated and extracts were analyzed by immunoblotting. Again, Ras was found in nuclear fractions (Fig. 3) of all stable cell lines transfected with *H-ras$^{Val\ 12}$* alone or in combination with temperature sensitive *p53$^{Val\ 135}$* or c-*myc*. Immunoblotting analysis with antibodies against the cytosolic farnesyl protein transferase β) (FPTase β and nucleic lamin A/C confirmed the degree of purity of nuclear fractions. As shown in Figure 3, cytosolic FPTase β was found exclusively in total cell extracts of control HeLa cells.

Nuclear Localization of Activated H-Ras$^{Val\ 12}$ and Posttranslational Modifications

Posttranslational modification of a C-terminal CAAX motif is necessary for generation of a hydrophobic C terminal domain in an otherwise hydrophilic molecule. Inhibition of the substrate specific farnesyltransferase has been shown to prevent further steps of processing [Dai et al., 1998] of the CAAX motif and to alter transforming activity of Ras [Booden et al., 1999]. If inhibition of farnesylation actually affects localization of the protein in the plasma membrane, then altering the modification status would be expected to change the distribution pattern of activated H-Ras$^{Val\ 12}$. To test this hypothesis, cells transformed with activated H-Ras$^{Val\ 12}$ and temperature sensitive p53$^{Val\ 135}$ were treated with farnesyltransferase inhibitor Cys-R-val-R-phe-m (Fig. 1). As depicted in Figure 1, H-Ras$^{Val\ 12}$ proteins were excluded from the nucleus in response to inhibition of

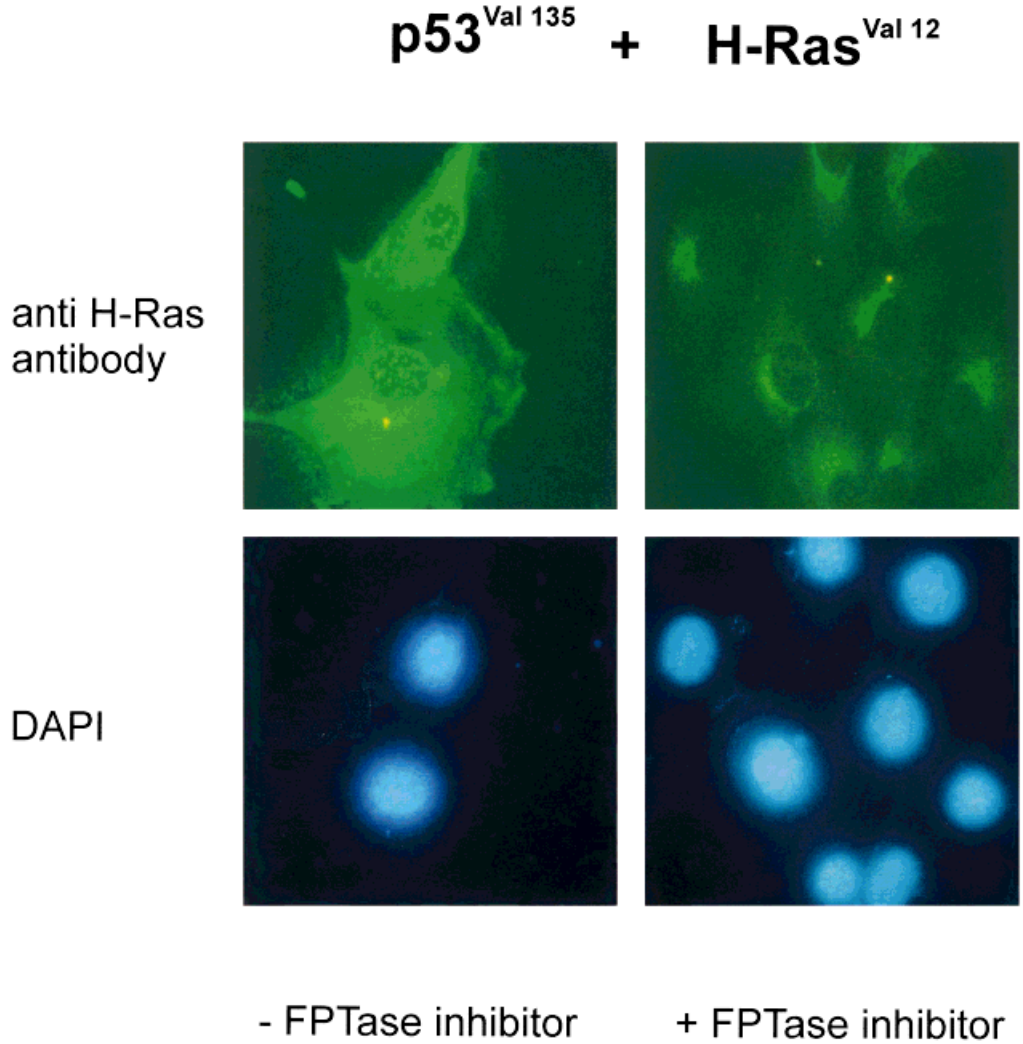

Fig. 1. Subcellular distribution of activated H-Ras$^{Val\ 12}$ analyzed by confocal microscopy. Cells were fixed with ice-cold methanol-acetone, permeabilized and labeled with anti-p21/c-H-Ras mouse monoclonal antibodies. Immune complexes were detected by incubation with Cy-2 fluorescein-conjugated anti–mouse IgG antibody. Nuclei were visualized with Dapi. Displayed are representative cells with distinct nuclear staining of three independent experiments.

prenylation. Moreover, the majority of cells treated with the inhibitor revealed a typical cytosolic pattern of fluorescence never observed in untreated cells. Indeed, immunofluorescence analysis revealed that both nuclear and cytoplasma membrane localization of oncogenic H-Ras$^{Val\ 12}$ depends on posttranslational modifications.

Effects of Farnesyltransferase Inhibitor Cys-R-val-R-phe-m on NFκB

Several lines of evidence assign NFκB to a crucial role in oncogenic Ras mediated cellular transformation [Norris and Baldwin, 1999] due to its anti-apoptotic function [Mayo et al., 1997]. With regards to conceivable functional repercussions in this context, specifically, rat embryonic cells transformed with activated H-Ras$^{Val\ 12}$ and temperature sensitive p53$^{Val\ 135}$ as well as normal RECs were treated with farnesyltransferase inhibitor Cys-R-val-R-phe-m or vehicle, respectively. Immunofluorescence analysis with anti-NFκB antibodies and counterstaining with Dapi revealed a distinctive pattern of cytoplasmic and nuclear localization (Fig. 4A) in untreated transformed cells. Interestingly, inhibition of farnesylation prevented translocation of NFκB into the nucleus of cells transformed with activated H-Ras$^{Val\ 12}$ and temperature sensitive p53$^{Val\ 135}$. In contrast to

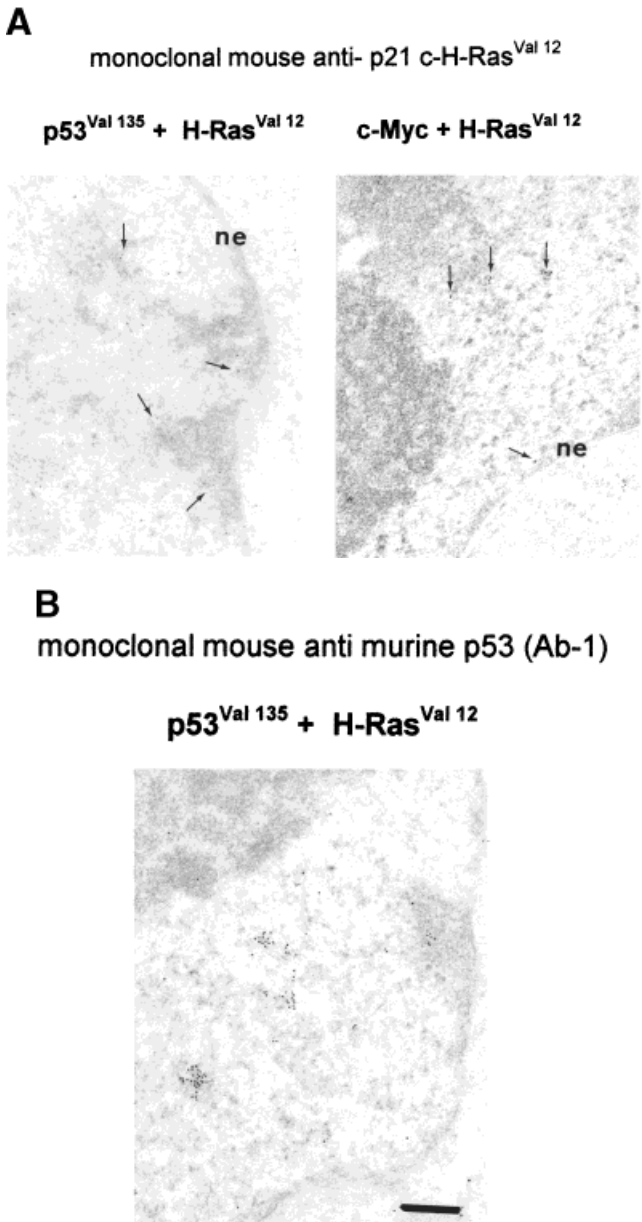

Fig. 2. (**A**) Electron microscopy of rat embryonic cells transformed with ts $p53^{Val\ 135}$ or c-Myc and activated H-$Ras^{Val\ 12}$. LR-White embedding and 5 nm colloidal gold immunostaining of Ras is represented. (**B**) Rat embryonic cells, expressing wild type p53 at permissive temperature (32°C) were used as control for nuclear localization. Arrows indicate specific staining of H-Ras (A) and $p53^{Val\ 135}$ (B). Bar = 250nm.

transformed cells, no effects of farnesyl transferase inhibitor treatment were detected in normal rat embryonic cells (Fig. 4B).

N-Nitrosodiethylamine Induced C3H Mouse Liver Tumors

Several studies have shown a correlation between mutational activation of H-Ras and tumor development in N-nitrosodiethylamine-induced hepatocarcinogenesis even though the expression of H-Ras mRNA did not alter during NDEA treatment [Giri and Das, 1996]. These findings suggest that additional mechanisms participate in NDEA-induced hepatocarcinogenesis. In an attempt to substantiate in vitro data concerning nuclear localization of mutant H-$Ras^{Val\ 12}$ in transformed primary rat embryonic cells we studied the subcellular distribution pattern of the oncoprotein in vivo. In a mouse

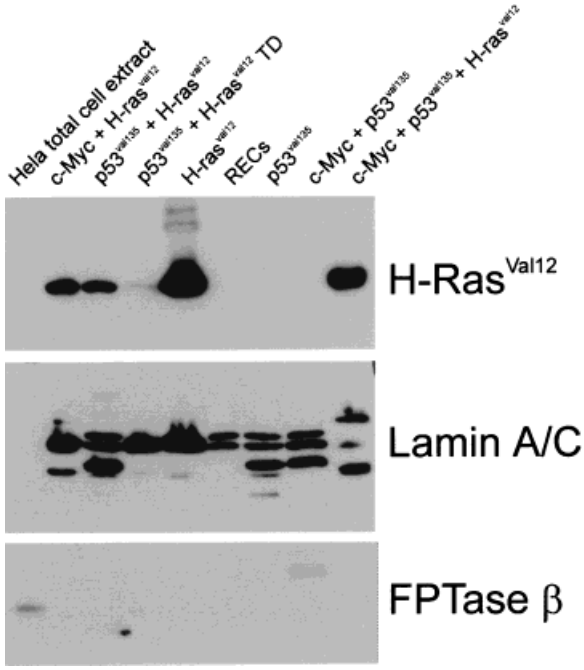

Fig. 3. Western blot analysis of nuclear extracts of cell lines derived from rat embryonic cells transfected with combinations of pVEJB (expressing activated H-Ras$^{Val\ 12}$ and the *neo* gene) and pLTRp53cG (ts p53$^{Val\ 135}$) or pSP*c-myc*. Nuclear extracts of p53$^{Val\ 135}$ + H-Ras$^{Val\ 12}$ tumor derived (TD) were prepared from tumors generated by injection of p53$^{Val\ 135}$ + H-Ras$^{Val\ 12}$ transformed cells in rats. For immunoblotting anti-p21/c-H-Ras antibodies derived from immunizing mice with recombinant c-H-Ras$^{Val\ 12}$ protein were used. To scrutinize nuclear fraction for cytosolic contamination immunoblotting with antibodies against farnesyl protein transferase β was performed. Presence of nuclear proteins was confirmed by sequential immunoblotting with monoclonal antibody against nuclear lamin A/C. The figure shows a representative experiment (n = 4).

model, NDEA induced tumors were characterized with respect to base substitutions in hotspot positions of the H-*ras* proto-oncogene [Frey et al., 2000]. Sections of liver tumors induced by NDEA and promoted by the hepatomitogen Phenobarbital in male mice revealed nuclear localization of oncogenic H-Ras (Fig. 5). Within the focus some nuclei exhibit specific staining (arrows) with anti-H-Ras antibodies (brown) and nuclei were counterstained with hematoxylin (blue).

Complex Formation With NTF2

The transport of proteins between the nucleus and the cytoplasm provides an effective circuit for regulation of gene expression. The initial observation concerning nuclear localization of activated H-Ras$^{Val\ 12}$ raised the possibility of participation of the oncoprotein in nucleocytoplasmic shuttling. To examine whether nucleic Ras acts like its relative, the GTPase Ran, which binds to nucleic NTF2, reciprocal immunoprecipitation experiments with anti-p21/ras and anti-NTF2 antibody was performed (Fig. 6). Samples obtained from immunoprecipitations with nuclear fractions of cells transformed with activated H-Ras$^{Val\ 12}$ and ts p53$^{Val\ 135}$ or oncogenic c-Myc were separated on SDS-PAGE and blotted on PVDF membranes. Indeed, nuclear H-Ras$^{Val\ 12}$ was found to interact with nuclear NTF2, as confirmed by immunoblotting (Fig. 6) with anti-NTF2 antibody. In reciprocal experiments the same results were obtained using NTF2 antibodies for immunoprecipitation and p21/ras antibodies for immunoblotting analysis (data not shown). Complex formation with nuclear NTF2 was found to be restricted to cells expressing activated H-Ras$^{Val\ 12}$ and due to nuclear localization of the oncoprotein. Regarding complexion of endogenous Ras and NTF2, there were no significant differences between normal rat embryonic cells and cells expressing mutant p53$^{Val\ 135}$ alone or in combination with oncogenic c-Myc

Subcellular Distribution of p27

It has been hypothesized that alterations in localization of p27^{Kip1} may represent an epigenetic mechanism for abrogating anti-proliferative cell cycle regulation [Reynisdottir and Massague, 1997; Orend et al., 1998] or Ras mediated apoptosis. To test this hypothesis in context with nuclear localization of activated H-Ras$^{Val\ 12}$, immunoblot analysis (Fig. 7) of subcellular fractions was performed. In cells expressing mutant p53$^{Val\ 135}$ and oncogenic H-Ras$^{Val\ 12}$, analysis of subcellular fractions revealed that expression of the phosphorylated form of p27 protein was different from the pattern observed in H-Ras$^{Val\ 12}$ and c-Myc transformed rat embryonic cells. Moreover, the level of phosphorylated p27 protein decreased in cells expressing nuclear H-Ras$^{Val\ 12}$ and oncogenic c-Myc compared to normal rat embryonic cells. Furthermore, cells transformed with a combination of the three oncogenes (mutant p53$^{Val\ 135}$; activated H-Ras$^{Val\ 12}$; and oncogenic c-Myc) revealed alterations of p27 protein levels. Additionally the non-phosphorylated form of p27 was observed to be the predominating form in contrast to cells transformed with H-Ras$^{Val\ 12}$ and complemented with mutant p53$^{Val\ 135}$. Degradation of p27

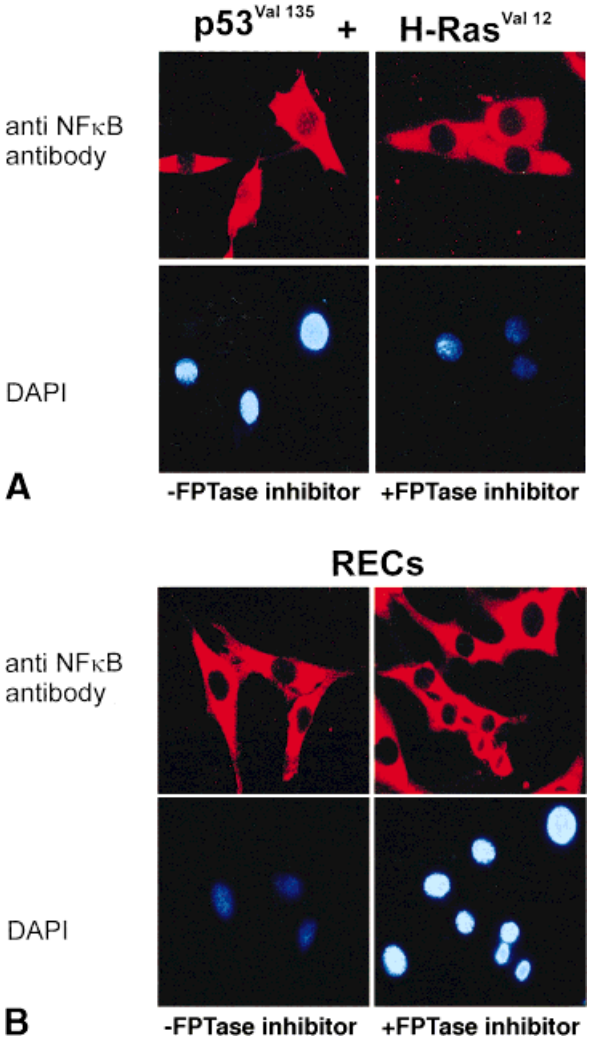

Fig. 4. Effect of FPTase inhibitor on distribution pattern of the transcription factor NFκB. RECs transformed with mutant c-H-Ras$^{Val\ 12}$ and ts p53$^{Val\ 135}$ (**A**) and normal rat embryonic cells (**B**) were processed for cytochemical detection of NFκB using anti-NFkB p65 antibodies. Nuclei were visualized with Dapi. Under control conditions NFκB was observed in the nucleus as well as in the cytoplasm of cells transformed with activated H-Ras$^{Val\ 12}$. In contrast, no alterations in the subcellular distribution pattern of NFκB were detected in normal RECs after treatment with the FPTase inhibitor. Prevention of nuclear targeting of NFκB in transformed cells after treatment with the inhibitor coincides with cytoplasmic localization of unmodified H-Ras proteins. Results are representative of four independent experiments.

requires import into the nucleus for phosphorylation and subsequent re-export to the cytoplasm for degradation. [Muller et al., 2000]. Accordingly, the distribution pattern of p27 was determined in subcellular fractions of cells possessing nuclear H-Ras$^{Val\ 12}$ (Fig. 7). Western blot analysis revealed that p27 was localized solely in the cytoplasm of transformed cells in contrast to nuclear localization of the cell cycle

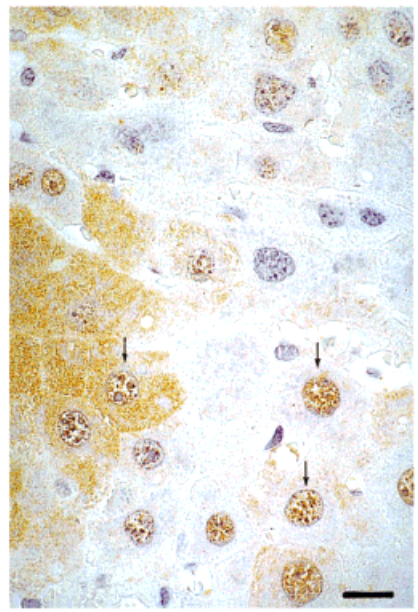

Fig. 5. Histochemical analysis activated H-Ras protein distribution pattern in liver sections from mice treated with NDEA and Phenobarbital. Paraffin-sections represent anti-p21/c-H-Ras immunoperoxidase-staining (brown) and hematoxylin nuclear counterstain (blue). Within the focus some nuclei exhibit specific staining (arrow). Bar = 20 μm.

inhibitor in normal rat embryonic cells. Moreover, the phosphorylated and non-phosphorylated forms of p27 were found to be restricted to the cytoplasm in cells cotransformed with mutant p53$^{Val\ 135}$. In contrast to that observation immunoblotting analysis of cytoplasmic fractions of H-Ras$^{Val\ 12}$ and c-Myc transformed cells revealed exclusively the non-phosphorylated form of p27 protein.

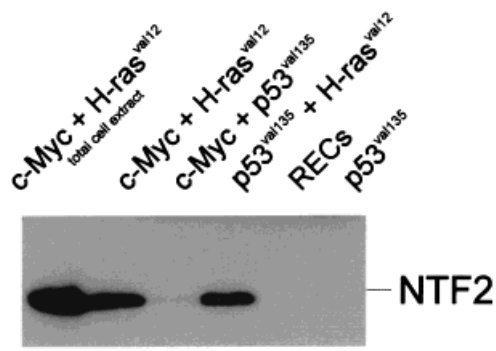

Fig. 6. Detection of NTF 2 protein in complexes immunoprecipitated from nuclear fractions with anti-H-Ras antibody. Immune complexes were electrophoretically separated and co-precipitated NTF2 protein was visualized by immunoblotting with anti-NTF2 antibody. NTF2 was detected in nuclear fractions of all H-Ras$^{Val\ 12}$ transformed cells clones. Results represented in this figure are of three independent experiments.

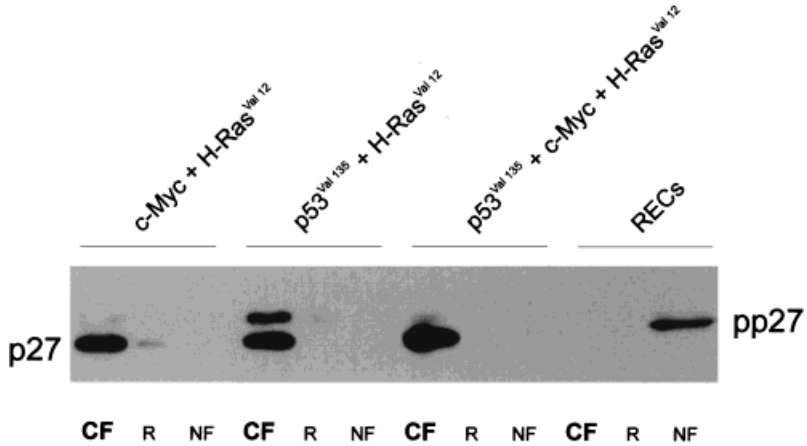

Fig. 7. Western blot analysis detecting p27 and phosphorylated p27 (pp27) in subcellular fractions of normal rat embryonic cells and RECs cotransformed with H-Ras$^{Val\ 12}$ and ts p53$^{Val\ 135}$ and/or c-Myc (NF-Nuclear Fraction / CF—Cytoplasmatic Fraction / R—total cell extracts). Data shown from three independent experiments.

DISCUSSION

Overexpressed H-Ras$^{Val\ 12}$ Resides in the Nucleus of Transformed Rat Embryonic Cells

Since targeting and stable localization of H-Ras proteins to the inner leaflet of the plasma membrane was suggested to be the unique prerequisites for the biological activity of the protein [Willumsen et al., 1996], there was an intense effort to investigate its subcellular distribution pattern. The discovery that at least two of the enzymes involved in posttranslational processing of the CAAX motif in the H-Ras protein were restricted to the endomembrane system [Dai et al., 1998] has recently provoked a revision of previous assumptions regarding the sites of sequential posttranslational modifications and transient subcellular distribution of H-Ras [Choy et al., 1999]. However, the results presented in this study, based on several distinct lines of evidence (Figs. 1, 3) indicate that mutant H-Ras$^{Val\ 12}$ can localize in the nucleus of transformed cells (Fig. 2A) in addition to its postulated cytosolic, plasma- or endomembrane localization. These data lead to the conclusion that nuclear localization of Ras represents an alternative, novel pathway where Ras signaling affects multiple targets within the nucleus.

Nuclear Localization of Activated H-Ras$^{Val\ 12}$ Depends on Posttranslational Modifications

In an attempt to understand the molecular mechanism that regulates subcellular distribution pattern of activated Ras proteins, specific farnesyltransferase inhibitors have been developed that selectively inhibit processing of these proteins [Omer and Kohl, 1997]. Treatment of Ras transformed cells with a specific farnesyltransferase inhibitor resulted in abrogation of cytoplasma membrane localization. Accordingly, posttranslational modifications have not been considered to be essential for nuclear localization of the protein. Surprisingly, the same inhibitor was able to exclude activated H-Ras$^{Val\ 12}$ proteins from the nucleus (Fig. 1). These data are implicative that farnesyltransferase inhibitors exercise an influence on both the cytoplasma membrane and nuclear associated functions of Ras. In accordance with regulatory effects on the growth of yeast depending on Ras2p localization [Boyartchuk et al., 1997; Tam et al., 1998] one might speculate that nuclear exclusion of Ras modulates rather than blocks Ras effector functions.

Farnesyltransferase Inhibitor Prevents Nuclear Targeting of NFκB

In addition to Ras, substrates of the farnesyl transferase include nuclear lamins, three proteins of the visual transduction system, skeletal muscle phosphorylase kinase, the peroxisomal protein Pxf, and the cell regulatory protein tyrosine phosphatases PTP_{CAAX1} and PTP_{CAAX2} [Gibbs and Oliff, 1997]. If inhibition of farnesylation affects particularly the intrinsic CAAX motives of those proteins (Fig. 1), one might predict that NFκB, which lacks this motif, would not be affected. Of particular interest is the finding that inhibition of farnesylation abrogated translocation of the transcription factor NFκB into the nucleus of transformed

cells (Fig. 4A). Interestingly, subcellular distribution pattern of NFκB was not altered in normal rat embryonic cells after treatment with the same inhibitor (Fig. 4B). The canonical mechanism for NFκB activation is based on the signal-inducible degradation of IκB, which in turn causes translocation of NFκB to the nucleus [Baldwin, 1996]. Since NFκB activity depends solely on its localization, nuclear exclusion of the protein after treatment with the farnesyltransferase inhibitor in transformed cells is suggestive for hampering transformation by oncogenic Ras in vitro and in vivo. However, the precise function of posttranslational modifications in this process remains to be elucidated.

Activated H-Ras Proteins Localize in the Nuclei of Tumors Induced by NDEA

Genetic evidence has been provided in a mouse model null for the tumor suppressor INK4a [Chin et al., 1999] that oncogenic Ras is crucial for both development and maintenance of tumors. Indeed, oncogenic H-Ras was found in the nuclei of NDEA induced liver tumors (Fig. 5) thereby supporting observations made in established cell lines transformed by H-Ras$^{Val\ 12}$ (Figs. 2A, 3). Based on these findings it is tempting to speculate that nuclear localization of oncogenic Ras occurs not merely as a passive consequence of overexpression upon transfection, but rather as an active homeostatic mechanism that participates in tumor development and maintenance.

NTF2 Associates With Nuclear H-Ras

Since the regulatory function of Ras depends on its subcellular distribution and the interaction with other proteins it was tempting to speculate about presumptive binding partners in context with nuclear localization. Precise bidirectional shuttling of macromolecules across the nuclear envelope requires the Ras-family GTPase Ran, nuclear transfer factor 2 (NTF2), and guanine triphosphate (GTP). Evidence for the involvement of another GTPase, distinct from Ras-related Ran, was presented by Sweet and Gerace [1996]. Detection of NTF2 protein in complexes immunoprecipitated from nuclear fractions with anti-H-Ras antibodies (Fig. 6) provided evidence that nucleic H-Ras$^{Val\ 12}$ protein might participate in nuclear-cytoplasmic shuttling. The GTP bound form of the Ras- relative Ran was shown to regulate cargo binding and release of import and export receptors in their respective target compartments and that transport through the nuclear pore complex can be reversed in the presence of high concentrations of Ran-GTP in the cytoplasm. [Nachury and Weis, 1999]. It is suggestive, that activated H-Ras$^{Val\ 12}$ located in the nuclei of transformed cells acts like the GTPase Ran determining directionality of nuclear transport. However, its specific contribution to nucleocytoplasmic trafficking of proteins remains to be determined.

Alteration in Subcellular Distribution of p27–Implications for an Alternative Form of Cell Cycle Regulation

Nuclear localization of activated H-Ras$^{Val\ 12}$ (Fig. 2A,B) constitutes a novel circumstance in which Ras might affect the distribution pattern of mediators involved in cell-cycle arrest. Analysis of the expression and subcellular distribution of p27^{Kip1} revealed that depletion of p27^{Kip1} in isolated nuclei coincided with nuclear targeting of activated H-Ras (Fig. 7). Since activation of p42/p44 mitogen-activated protein (MAP) kinase cascade is commonly targeted in H-Ras$^{Val\ 12}$ transformed cells, these findings imply that nucleic H-Ras$^{Val\ 12}$ proteins may facilitate S-phase entry through mechanisms involving cytoplasmic retention or active nuclear export of p27. Interestingly, the mechanism of p27 regulation was mediated by both cytoplasmatic localization and phosphorylation status of the protein (Fig. 7). However the level of p27 was altered in cells possessing nuclear Ras and mutant p53$^{Val\ 135}$. In contrast, cells cotransformed with H-Ras$^{Val\ 12}$ and oncogenic c-Myc, revealed downregulation of the phosphorylated form of p27 (Fig. 7), suggesting that there is some p53 dependency on phosphorylation of the cell cycle inhibitor. These data lead to the conclusion that association of nucleic NTF2, involved in nuclear export [Englmeier et al., 1999] and oncogenic H-Ras$^{Val\ 12}$ could contribute to regulation of p27 activities by altering the subcellular distribution pattern of the cyclin dependent kinase inhibitor (Fig. 7).

CONCLUSIONS

This work primarily focused on the intracellular distribution of activated H-Ras oncoprotein and herein reports for the first time the nuclear localization of the protein and its

complex formation with nuclear NTF2. These findings underscore the pleiotropic nature of intracellular signaling of activated H-Ras$^{Val\ 12}$ and may provide a mechanism to suppress apoptosis and maintain oncogenic activation.

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