Levels and membrane localization of the c-K-ras p21 protein in lungs of mice of different genetic strains and effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and Aroclor 1254

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Introduction

Among human lung cancers, adenocarcinomas have been increasing in frequency (1) and at least 30% of these contain a mutated K-ras oncogene (2,3). These mutations are particularly likely in smoking-related adenocarcinomas (4) and are associated with poor prognosis (5–7). In addition to causing genotoxic mutational events, cigarette smoke constituents and other exposures may contribute to lung cancer development by participating in tumor promotion. The reduction in cancer risk by cessation of smoking and the protective effect of dietary components containing anti-oxidants are consistent with this hypothesis. It has, however, been little explored with animal models. Although 6 months exposure to toxic levels of cigarette smoke failed to promote lung tumors initiated in A/J mice in a recent study, desirability of longer exposure and/or non-toxic doses was noted by the authors (8).

The adenomatous primary lung tumor of the mouse is an excellent model for some forms of human lung adenocarcinoma, as it is morphologically similar, and also presents mutated K-ras oncogenes with high frequency (9). These tumors are readily initiated by chemical carcinogens such as are found in cigarette smoke, including polycyclic aromatic hydrocarbons and nitrosamines. They also are subject to tumor promotion, by butylated hydroxytoluene (BHT*) (10), polychlorinated biphenyls (PCBs) (11–13), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (14) and glycerol (15). Understanding of the mechanism by which these mouse lung tumors are promoted could indicate whether promotion is an important part of lung adenocarcinoma tumorigenesis in humans, as well as reveal possible targets for intervention. Since K-ras is frequently and site-specifically mutated in mouse lung tumors that arise after treatment with genotoxicants, a reasonable hypothesis is that the tumor promoters either enhance the expression or efficacy of the mutated K-ras allele or, alternatively, functionally mimic the mutated K-ras p21 and co-operate with other, as yet undefined, genetic change(s).

Little information is available bearing on these hypotheses. Mouse lung tumors initiated by the tobacco-specific 4-(methyl-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and promoted by BHT presented a lower percentage of K-ras mutations than those arising after NNK only (16). A similar reduction in frequency of H-ras mutations was noted for mouse liver tumors promoted by various treatments (17–19). On the other hand, mouse liver tumors promoted by TCDD (20) and mouse lung tumors promoted by TCDD or PCBs (21) had higher incidences of H- or K-ras mutations, respectively, than those resulting from initiator-only treatment. It is possible if not likely that promoters of different chemical type operate through distinct mechanisms.

Since mutant ras p21 is thought to have its effect due to an abnormally high rate of signal transmission through the cell membrane (22), the functionality of either the normal or the mutant allele and protein could be enhanced by increased amounts of the p21 protein in the membrane. As a first step in examining such a possible increase as a contributing factor in lung tumorigenesis, we have developed a method for quantitative determination of K-ras p21 in mouse lung tissue fractions, tested for differences among mouse strains, and examined the effects of tumor-promotive doses of TCDD and PCBs. We have found that lungs from mice of different genetic strains, while containing similar total amounts of K-ras p21, show marked variation with regard to amount in the membrane fraction. Furthermore, TCDD and PCBs can increase both total amount of K-ras p21, and the membrane/cytosol ratio, but only in certain mouse strains.

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Materials and methods

**Chemicals**

TCDD (99% pure) was purchased from the Midwest Research Institute (Kansas City, MO). Olive oil and other chemicals were obtained from Sigma Chemicals. v-H-ras agarose conjugated antibody and c-K-ras antibody (OP24) were obtained from Oncogene Science, Cambridge, MA. ECL kits were purchased from Amersham. Arocrol 1254 was obtained from Anlabs Inc., North Haven, CT.

**Animals and treatment**

Male NIH Swiss, C57BL/6, BALB/c, DBA and AKR mice were obtained from North Haven, CT. Animals and treatment were obtained from Oncogene Science, Cambridge, MA. ECL kits were purchased from Amersham. Arocrol 1254 was obtained from Anlabs Inc., North Haven, CT.

**Materials and methods**

**B**protein), containing K-ras p21. Lane 4, mouse lung lysate treated with agarose conjugated to non-specific IgG. The 21 kDa band is absent. (Lane 1, Anti-ras-agarose alone (negative control). Lane 2, human recombinant ras p21 standard (positive control). Lane 3, mouse lung lysate (250 µg of protein amounts is shown for membrane fractions from control and TCDD-treated (C57BL/6) lungs. The amounts of protein indicated were represented the total lysate. The other half was subjected to ultra centrifugation by resuspension in the homogenizing buffer and re-centrifugation, and then solubilized in membrane detergent lysis buffer (10 mM Tris-HCl, pH 7.4, 1% Triton X100, 0.1% SDS, 1.5 mM NaCl, aprotinin and PMSF). Protein contents were determined by BCA reagent (Pierce, Rockford, IL).

**Immunoprecipitation and Western blotting**

Frozen lung tissues were homogenized in hypertonic buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 10% sucrose, aprotinin and PMSF. The homogenate was centrifuged at 10000 g at 4°C and the supernatant was divided into different subcellular fractions. To one half of the supernatant 1/10th volume of 5 x 10^6 lysis buffer [homogenization buffer containing 20 mM HEPES (pH 7.4), 1 mM EDTA, 1 mM MgCl2, 1 mM DTT and protease inhibitors phenylmethylsulfonylfluoride (PMSF), 1 mM, and aprotinin (0.2 TIU)]. The cells were homogenized and the membrane and cytoplasmatic fractions were separated as described below.

**Immunoprecipitation**

**Western blotting**

The immunoprecipitate was washed three times in the wash buffer and resuspended in 2X SDS sample buffer containing 400 mM diethiothreitol (DTT). Samples were run on 12% polyacrylamide gels (Novex) at 100 V and transferred to Hybond nitrocellulose membrane at 200 mA at 4°C. The blot was blocked in 10% dry milk overnight at 4°C and probed with K-ras monoclonal antibody (OP24). The blots were incubated for 60 min at room temperature in secondary antibody (horseradish peroxidase labeled goat anti-mouse made in 1% gelatin, Amersham). After repeated washes in TBS containing 0.5% Tween-20 the blots were developed using a chemiluminescence ECL kit and exposed to X-ray film. At least four animals were used, from each of the six strains, for comparison of p21 from total, membrane and cytosolic fractions, and for most samples the assays were repeated a second time.

**Densitometric analysis of band intensity**

To quantify the levels of p21 ras in the cytosolic and membrane compartments the total intensity of each band obtained was determined using a densitometer scanner (Molecular Dynamics, Inc.), and corrected for background. The integration values for each band were taken as arbitrary units and calculations made relative to a reference band, as described in the text. Parametric and non-parametric statistical tests were carried out as appropriate (Instat, Inc. software program).

**Results**

**Specific detection and quantification of c-K-ras p21 in mouse lung**

To increase the sensitivity of the assay and to reduce the background of nonspecific reactivity, we first immunoprecipitated the p21 from cellular extracts using agarose to which a murine anti-H-ras p21 was conjugated, before gel electrophoresis and probing with c-K-ras p21-specific monoclonal antibody. The positive control used was purified recombinant human K-ras p21. A 21 kDa protein was recovered from mouse lung homogenate by this method, with no bands in this region for negative controls (ras-agarose in the absence of tissue, and tissue plus agarose conjugated to a nonspecific IgG) (Figure 1A). Non-specific bands observed in the range of 28–52 kDa correspond to the light and heavy chains of immunoglobulin (Figure 1B). The amount of agarose used (30 µg protein/ml lysate) was sufficient to recover all of the p21 with good reproducibility. This was determined in a separate set of experiments in which 10–40 µg Ras-conjugated agarose antibody protein was used. The supernatant resulting from this step was further immunoprecipitated with the ras-agarose conjugated antibody to check for the complete precipitation of the ras. Although 10 µg was not completely sufficient, 20–40 µg of the antibody was sufficient to precipitate all the ras p21 from the lysate (data not shown). The method was quantitatively approximately linear in the range of 100–250 µg protein loaded (Figure 1C).

**Levels of total K-ras p21 in lungs of mice of different strains**

Total lung p21 protein from four mice of each of the six mouse strains was compared in three blots and quantified by densitometric scanning. No consistent differences were observed (Figure 2).

**Membrane/cytosol distribution of K-ras p21 in lung of mice of different strains**

Lungs of two mice of each strain were separated into membrane and cytosolic fractions and analyzed in two immunoblots.
Fig. 2. Comparison of total p21 ras in lung tissue from different strains of mice. This blot is representative of three assays of a total of four mice of each strain. For each lane 250 µg protein equivalent of total lung lysate immunoprecipitate was loaded as indicated. Densitometric scan values of the resulting p21 protein bands confirmed similar levels in all strains.

Fig. 3. Amounts of p21 ras in membrane and cytosolic fractions. This blot and the corresponding densitometric scan is representative of two assays. 250 µg protein of membrane fraction and 500 µg of cytosolic fraction were immunoprecipitated and loaded as indicated.

(Figure 3). Membrane bound K-ras p21 was consistently greater in amount than that in cytosol. Ratio of amounts in the membrane and in the cytosolic fraction were calculated from densitometric scans after correction for differences in amounts of protein loaded. Substantially more K-ras p21 was found in the membrane fraction compared with the cytosolic fraction, in C57BL/6 lungs (ratios of 3.8 and 14.8) and in BALB/c lungs (7.3 and 2.8). This ratio was somewhat lower in Swiss lungs (3.3 and 2.3). The two DBA lungs varied (ratios of 5.2 and 0.4). Of particular interest was the fact that the AKR mice had low membrane/cytosol ratios (1.2 and 1.7), whereas the AKR.B6Ah congenic mice had high ratios, similar to those in C57BL/6 mice (10.0 and 12.7).

These interesting differences were further pursued by comparing four mice of each strain, using separate blots for membrane and cytosolic fractions. A representative membrane fraction blot is shown in Figure 4A. The membrane content of K-ras p21 was consistently high in C57BL/6 lungs. For the densitometric scans of each blot, the higher C57BL/6 value was assigned a value of 1.0 and the other values calculated as a percentage. The results and statistical comparisons are shown in Figure 4B. BALB/c lung membrane fractions also contained high levels of p21, as did those of AKR.B6Ah mice, although the AKR.B6Ah congenic mice had high ratios, similar to those in C57BL/6 mice (10.0 and 12.7).

Fig. 4. Comparison of amounts of p21 ras in membrane fractions from mice of different strains. Lung membrane fractions were prepared from four mice of each strain and compared in three blots; a representative blot is shown in (A). Densitometric scan values were converted into ratios based on the highest value for a C57BL/6 lung on the blot. These ratios were then averaged and utilized for statistical analysis (B). Values with the same superscript are significantly different as follows. Tukey–Kramer Multiple Comparison Test, 2-tailed unless indicated: a,b P < 0.01; c,c P < 0.001; d,d P = 0.057, one-tailed; e,e P = 0.019. Pairwise two-tailed test: f,f P < 0.0001; g,g P < 0.01.

Fig. 5. Representative immunoblots of lung cytosolic fractions. K-ras p21 was detected in each cytosolic fraction, but the relative amounts in different individual lungs and in lungs of mice of different strains were variable.

limitations of the method with cytosols containing low amounts of the protein.

Effects of TCDD and PCBs on total and membrane/cytosol ratios of K-ras p21

TCDD had strain-specific effects. In C57BL/6 mice, TCDD treatment caused a significant, 2–3-fold increase in K-ras p21 in lung total lysate and in the membrane fraction, with no change in cytosol and on average a 3-fold increase in membrane/cytosol ratio (Figure 6A). In BALB/c mice, the total K-ras p21 levels were unchanged by TCDD, but a significant increase in membrane p21 and a decrease in cytosol p21 resulted, with an average doubling in membrane/cytosol ratio (Figure 6B). A similar trend was noted for DBA/2 mice, but only the increase in the membrane fraction was of statistical significance; the membrane/cytosol ratio was doubled (Figure 6C). For the Swiss mice again a significant increase in the membrane fraction was seen, though of small magnitude, and
Fig. 6. Effects of TCDD treatment on total, membrane, and cytosol localization of K-ras p21. Four mice of each strain were treated with TCDD or oil and killed after 48 h, and total lung lysates, membrane (MEM) and cytosol (CYT) fractions assayed as described. The bar graphs represent the densitometric scan values. $P$ values shown are from the Student’s $t$-test or Welch’s test as appropriate.
Control

TCDD

p21

21 kD

T M C T M C

Fig. 7. K-ras p21 in cultured E10 cells. A representative experiment is shown. Cultures at 70–80% confluence were serum-starved (0.1% serum) for 24 h, then given fresh medium with 10% serum, and 10 nM TCDD or DMSO, and harvested at confluence 24 h later. T, total; M, membrane; C, cytosolic fractions.

results for both the total lysate and the cytosolic fraction were variable, with a small (30%) overall increase in membrane/cytosol ratio (Figures 6D). For both the AKR and the congenic AKR.B6Ah mice, TCDD had no effect on the level of K-ras p21 in any fraction (Figures 6E and F).

The PCBs mixture Aroclor 1254 was tested only in Swiss mice, where it has been repeatedly found to promote nitrosamine-initiated tumors (11–13). Lungs from four male mice were assessed 48 h after exposure to 250 mg/kg Aroclor 1254, with total lysate, membrane and cytosolic K-ras p21 from the eight mice quantified on separate blots (not shown). Considerable variation was noted; there were nonsignificant increases in total protein (relative densitometric scan values: 223.4 ± 79 and 365 ± 284 for controls and PCBs-treated mice, respectively) and in membrane protein (564 ± 286 versus 642 ± 277). However, cytosolic protein was significantly reduced after PCBs treatment (286 ± 52 versus 177 ± 36, P = 0.03), and membrane/cytosol ratios significantly increased (2.0 ± 0.7 versus 4.3 ± 1.1, P = 0.01).

K-ras p21 in E10 cultured lung alveolar cells

The above studies were carried out with whole lung, which contains numerous cell types, whereas the target cells for tumorigenicity include the alveolar type 2 cells. In cultured E10 immortalized alveolar type 2 cells, K-ras p21 was localized primarily in the membrane fraction When subconfluent cultures were serum-starved for 24 h and then treated with 10 nM TCDD in the presence of serum, a 4-fold increase in membrane K-ras p21 was observed after 24 h compared to the serum stimulated cells containing DMSO only (Figure 7).

Discussion

Normal levels of the ras protooncogene protein isoforms vary among tissues and as a function of age. In adult rats, K-ras p21 levels were highest in brain and lung, and in ovary varied with age and reproductive status: quantities in ovary were greatest during pregnancy and lactation and lowest in newborns (25). Similarly K-ras p21 amounts increased with postnatal maturation in gut and liver of mice as measured by RNA analysis (26) and in lungs of rats studied by Western blot and immunohistochemistry (27). These findings suggest a role for K-ras p21 in differentiated tissue functioning. On the other hand, selective over-expression of mutant K-ras alleles or protein has been observed in human tumors of several types (28,29) and in mouse lung tumors (30). These various reports suggest that total amount of K-ras p21 could be an important variable impacting on cancer development. However, this variable has been little examined as a function of genetics.

In our comparisons of K-ras p21 in lungs of mice of different strains, total K-ras p21 was relatively constant, although some individual variation was noted. Differential expression has been reported of K-ras alleles in (A/J × C3H)F1 and (A/J × TSG1-p53)F1 hybrid mice (30–32). The A/J allele was the more highly expressed in normal lung tissue (2–5-fold) and in lung tumors (up to 12-fold) in these hybrid mice. These results do not necessarily conflict with our findings, which do not as yet include A/J and C3H mice. Post-transcriptional control of K-ras p21 and/or variations in this parameter related to mouse husbandry in different animal facilities, are also possibilities.

Ras p21 proteins are targeted to cell membranes by posttranslational farnesylation and carboxy methylination. H-ras, N-ras and K-ras-4A proteins are also palmitoylated, whereas K-ras-4B lacks a palmitoylation site, but instead possesses a polylysine domain that is essential for its membrane localization (33,34). The physiological reasons for this distinct protein design are not known but could indicate the presence of specific localization sites in the membrane membrane/cytosol ratio in skin tumors (35); a single protein band was detected from both membrane and cytosol fractions. Ours appears to be among the first studies of K-ras-4B p21. Experiments with COS cells and their subcellular fractions have shown that fully processed K-ras(B) p21 automatically localized to the cell membrane, but that some cell transformation still occurred with mutant proteins not associated with membrane (33). H-ras p21 was found to be more firmly associated with the membranes of COS cells than K-ras-4(B) p21 (34).

Furthermore studies of epithelial tissues have shown significant amounts of ras p21 in the cytosolic fractions. In the normal epidermis of mice the majority of H-ras p21 was cytosolic, with an increase in total amount and in the membrane/cytosol ratio in skin tumors (35); a single protein band was detected from both membrane and cytosol fractions. Ours appears to be among the first studies of K-ras p21 membrane/cytosol ratios in epithelial cells. As for H-ras p21 in mouse skin (35), we found a single K-ras p21 band in cytosols and membrane fractions, usually at lower levels in the cytosol than in the corresponding membrane fraction.

The relative amounts of K-ras p21 in lung membrane and cytosol varied between strains and, for some strains, among individuals. This suggests that membrane targeting of the protein is controlled by both genetic and epigenetic factors, and is a variable that could, potentially, contribute in an important way to lung cancer susceptibility and could be utilized for prevention or therapy. Indeed, efforts are already underway to suppress lung cancers by treatment with agents that block farnesylation and hence membrane localization of mutant K-ras p21 (36,37). Our assays were carried out with whole lung, which consists of numerous different cell types. However, a recent immunohistochemical study with rat lung showed that K-ras p21 expression occurs only in the alveolar lining cells (27); confirmation of this important result is possible only in the mouse lung tissues is in progress in our laboratory. Further confirmation of the pertinence of our whole-lung findings for type 2 cells was provided by results with the cultured E10 cells. The membrane/cytosol ratio of K-ras p21 in these BALB/c-derived cells was similar to that in whole BALB/c lung.

The genetic factors that control K-ras p21 membrane localization are still unknown. Our studies with mouse strains suggest that the level of K-ras p21 is variable and could be critical in determining cancer susceptibility. Further work is underway to suppress lung cancers by treatment with agents that block farnesylation and hence membrane localization of mutant K-ras p21 (36,37). Our assays were carried out with whole lung, which consists of numerous different cell types. However, a recent immunohistochemical study with rat lung showed that K-ras p21 expression occurs only in the alveolar lining cells (27); confirmation of this important result is possible only in the mouse lung tissues is in progress in our laboratory. Further confirmation of the pertinence of our whole-lung findings for type 2 cells was provided by results with the cultured E10 cells. The membrane/cytosol ratio of K-ras p21 in these BALB/c-derived cells was similar to that in whole BALB/c lung.
ation may include the Ah receptor. Membrane levels of the protein were highest in the highly Ah-responsive inbred strains, C57BL/6 and BALB/c, and consistently lower in the less responsive (Ah<sup>b</sup>) DBA/2 and AKR mice. Importantly, the congeneric AKR.B6Ah, with the Ah<sup>b–1</sup> allele on an AKR genetic background, had low membrane levels of K-ras p21 that were between those of the parent strains, significantly more than AKR, but less than C57BL/6. This observation suggested that Ah<sub>r</sub> is among the genetic factors influencing membrane localization of K-ras p21.

In pursuit of this putative role of the Ah receptor, we examined the effects of the potent Ah receptor agonist and lung tumor promoter, TCDD. This compound indeed had significant, strain-specific effects, but these did not have an obvious relationship to the Ah<sub>r</sub> genotype. In C57BL/6 mice, there was a significant doubling in total lung K-ras p21 in response to TCDD and a 3-fold increase in the membrane fraction. Previous studies have shown that TCDD and PCBs upregulate H-ras levels in rodent hepatic tissues (38–40). Direct effects on gene expression are possible, as a Dioxin Response Element consensus sequence, GCCTG, is present starting at base 331 in the 5′ promoter region of the mouse c-K-ras gene (41) and in the 3′ untranslated region starting at base 1158 (30).

Although this response of the C57BL/6 mice to TCDD appeared to provide additional evidence implicating the Ah receptor, results with the other strains suggested a more complicated scenario. Ah-responsive BALB/c and non-responsive DBA/2 mice both showed significant shifting of K-ras p21 from cytosol to membrane in response to TCDD. Data were more variable from the Swiss mice, but an increase in membrane-localized K-ras p21 was still of statistical significance. Ah non-responsive AKR mice showed no changes in K-ras p21 after TCDD, and the presence of the Ah<sub>b–1</sub> allele in the AKR congenics did not confer sensitivity to TCDD. Thus, TCDD had marked effects on K-ras p21 in lung of mice of certain strains, consistent with stimulation of this signaling pathway as part of the promotion mechanism. It is possible that TCDD and/or the TCDD/Ahr complex directly affects the membrane localization of K-ras p21 by changing membrane properties or by modulating the cytosolic interactions of p21. Immediate effects of activated Ah receptor on protein phosphorylation and on cell cycle regulation, independent of altered gene transcription, have been reported (42,43). Recent studies indicate potential links between both the Ah receptor (44) and ras p21 (45) and progression of the cell cycle. Whether these proteins interact in this context is an important question for future study.

Genetic differences between the mice strains are major determinants of susceptibility to lung tumor initiation. Of the strains used in our study, BALB/c and Swiss are intermediate in susceptibility, while C57BL/6, DBA and AKR are resistant strains (46). The genetics of susceptibility to mouse lung tumor promotion has been little studied, but are known to be distinct from those of sensitivity to initiation (46). BALB/c mice, which had high levels of membrane K-ras p21, are especially responsive to lung tumor promotion by BHT (47). We have found that TCDD and PCBs are effective promoters of nitrosamine-initiated lung tumors in Swiss mice (11–14). A comparative study of these chlorinated compounds in mouse of other strains is desirable, especially in view of data implicating TCDD in causation of human lung cancer (48).

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