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Biochemistry

Effects of two major activating lesions on the structure and conformation of human *ras* oncogene products

(p21 proteins/amino acid substitutions/transforming genes/intramolecular disulfide bonds)

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ABSTRACT ***ras* oncogenes are frequently activated in human tumors by mutations at codon 12 or 61 in their coding sequences. To investigate how these subtle alterations exert such profound effects on the biologic activities of these genes, we studied structural and conformational properties of human *ras*-oncogene-encoded 21-kDa proteins (p21s). We observed striking differences in the electrophoretic mobilities of the proteins under reducing and nonreducing conditions. These findings imply that intramolecular disulfide bonds affect native p21 conformation. The two activating lesions were shown to induce distinctly different alterations in p21 electrophoretic mobility that were unmasked only under reducing conditions. These results suggest that regions of the molecule containing such alterations are either not exposed or under conformational constraints in the native p21 molecule. We confirmed the opposing effects on protein mobility induced by the two activating lesions by using a recombinant gene containing both lesions. The recombinant gene's high-titer transforming activity further established that the two lesions do not negatively complement one another with respect to transforming-gene function. Our findings of distinct alterations in electrophoretic mobilities of position-12- and position-61-altered p21 molecules should be applicable to the rapid immunologic diagnosis of *ras* oncogenes in human malignancies.**

DNAs from a wide variety of human tumors have been shown to contain transforming DNA sequences detectable upon transfection of NIH/3T3 cells (1–9), a continuous mouse cell line that is contact-inhibited and highly susceptible to the uptake and stable integration of DNAs (10, 11). A large majority of the transforming genes detected by this approach are derived from a small family of evolutionarily conserved genes, designated *ras* (9, 12–18). The human cellular *ras* family consists of three proto-oncogenes, H-*ras*, K-*ras*, and N-*ras*, which are closely related at nucleotide-sequence and protein-coding levels (19, 20). H- and K-*ras* were initially detected as the cell-derived *onc* genes of Harvey and Kirsten murine sarcoma viruses (MSVs), respectively (21, 22). No viral counterpart has yet been found for N-*ras*, which was initially isolated as an oncogene from a human neuroblastoma cell line (20).

ras oncogenes, whether of viral or cellular origin, encode 21-kDa proteins (p21s) (23). Investigation of the properties of Harvey- and Kirsten-MSV p21s has revealed that they associate with the cell membrane (24), bind guanine nucleotides (25), and undergo autophosphorylation using GTP as substrate (26, 27). While the normal cellular functions of *ras* proteins are not known, the genetic lesions responsible for activation of a number of human *ras* oncogenes have been precisely localized to single-base changes in their p21 coding sequences (28–36). Moreover, mutations at codons 12 or 61 appear to be major "hot spots" for activation of these genes under natural conditions. The high prevalence of *ras* oncogenes in human tumors has given impetus to efforts aimed at elucidating the *ras* gene function(s). The present studies were undertaken to investigate the effects of lesions at positions 12 and 61 on the structure and conformation of p21 proteins encoded by different human *ras* oncogenes.

MATERIALS AND METHODS

Cell Lines. The sources of human tumor cells and of tumor-cell DNAs used to obtain NIH/3T3 transformants by transfection are described in Table 1.

Metabolic Labeling. Cells were plated at 1–2 × 10^6 cells per 100-mm dish. For [^{35}S]methionine labeling, the growth medium was removed and the cells were incubated with methionine-free Dulbecco's modified Eagle's medium (Met^-DME) for 30 min. After removing Met^-DME, cells were incubated for 3–4 hr with Met^-DME (3 ml per dish) containing [^{35}S]methionine (New England Nuclear; >1100 Ci/mmol, 1 Ci = 37 GBq) at a concentration of 300 μCi/ml. For [^{32}P]orthophosphate labeling, the cells were incubated first with phosphate-free DME and then with phosphate-free DME (3 ml per dish) containing [^{32}P]orthophosphate at a concentration of 166 μCi/ml.

p21 Immunoprecipitation and $NaDodSO_4$/PAGE. The procedures were as previously described (37). Cells were extracted with 1% Triton X-100/0.1% $NaDodSO_4$/0.5% sodium deoxycholate/0.1 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM phenylmethanesulfonyl fluoride/aprotinin (100 kallikrein inactivator units per ml). Extracts were cleared at 4°C by centrifugation at 45,000 rpm for 30 min in a type 65 Beckman rotor and then either used immediately for immunoprecipitation analysis or stored at −70°C. Each immunoprecipitation sample contained 2–4 × 10^7 trichloroacetic acid-precipitable cpm for ^{35}S-labeled extracts or 1 × 10^7 cpm for ^{32}P-labeled extracts. Cell lysates were incubated for 30 min at 4°C with 15–20 μl of anti-Harvey-MSV-p21 monoclonal antibodies (YA6-172 or Y13-259) (38). [YA6-172 and Y13-259 hybridoma lines were provided by E. M. Scolnick (Merck Sharp & Dohme Research Laboratories).] Protein A-Sepharose was swollen and washed as described (37) and coated with goat or rabbit anti-rat IgG by the method of Furth *et al.* (38); 100 μl of a 1:10 (wt/vol) suspension of the coated protein A-Sepharose was added to each sample, and the samples were shaken in an Eppendorf shaker at 4°C for 15 min. The immunocomplexes were washed and the final pellets were dissolved at 90°C for 2–3 min in $NaDodSO_4$/PAGE sample buffer either containing 4.25% (vol/vol) 2-mercaptoethanol for reducing gels or without mercaptoethanol for nonreducing gels.

$NaDodSO_4$/PAGE of immunoprecipitated samples was done in 12% polyacrylamide slab gels with a Tris/glycine buffer system (37). For fluorography, gels were treated with EN^{3}HANCE (New England Nuclear) before drying. Dried

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Abbreviation: MSV, murine sarcoma virus.

Table 1. Mutations leading to activation of *ras* oncogenes in human tumor-derived cell lines

Cell line	Origin	Oncogene	Codon affected	Amino acid change	Ref.
T24	Bladder carcinoma	H-*ras*	12	Gly→Val	29
HS578T	Mammary carcinosarcoma	H-*ras*	12	Gly→Asp	36
Hs242	Lung carcinoma	H-*ras*	61	Gln→Leu	32
SW1271	Lung carcinoma	N-*ras*	61	Gln→Arg	35
JPT26	Renal pelvic carcinoma	H-*ras*	61	Gln→Arg	39, *
A427	Lung carcinoma	K-*ras*	ND	ND	8
Calu-6	Lung carcinoma	K-*ras*	ND	ND	*

ND, not determined.
*Our unpublished observations.

gels were exposed to x-ray film at −70°C for the desired time.

RESULTS

Heterogeneity in Electrophoretic Mobilities of p21 Molecules Encoded by Human *ras* Oncogenes. The p21 products of representative H-*ras*, K-*ras*, and N-*ras* human oncogenes were analyzed for their electrophoretic mobilities on NaDodSO$_4$/polyacrylamide gels. The human tumor cells used and the lesions activating their respective *ras* oncogenes are listed in Table 1. Cells were labeled with [^{35}S]methionine and analyzed for p21 expression by immunoprecipitation with monoclonal antibody (Y13-259) prepared against Harvey-MSV p21 (38). As shown in Fig. 1, some of the tumor cell lines (lanes c, g, and i) showed levels of p21 that were 2- to 4-fold higher than that observed with normal human fibroblasts (lane a). The highest p21 level was consistently observed with the T24 bladder carcinoma line (lane c). Monoclonal antibody YA6-172, also prepared against Harvey-MSV p21 (38), did not recognize the p21 protein encoded by members of the activated N-*ras* gene family, although this antibody readily detected the translational products of H-*ras* and K-*ras* human oncogenes (data not shown). The immunoprecipitation of p21 molecules encoded by human K-*ras* oncogenes with the YA6-172 monoclonal antibody contrasts with its reported inefficiency in recognizing the p21 of Kirsten MSV (38).

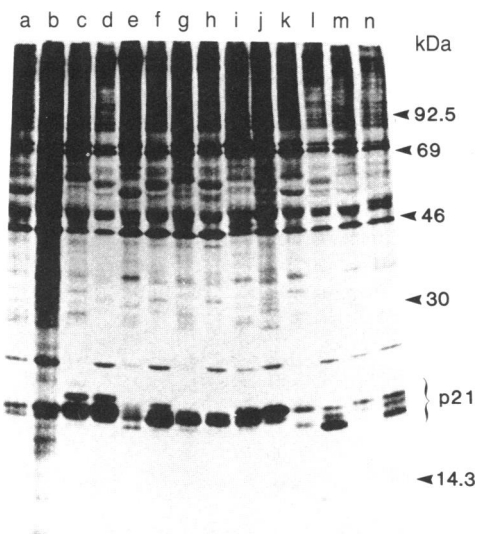

FIG. 1. Heterogeneity in the electrophoretic mobilities of human *ras*-oncogene-encoded p21 proteins. [^{35}S]Methionine-labeled cell extracts from human tumor-cell lines and NIH/3T3 transfectants containing oncogenes derived from each tumor line were immunoprecipitated with p21 monoclonal antibody Y13-259 and analyzed by NaDodSO$_4$/12% PAGE and fluorography, as described in *Materials and Methods*. Lane a, 501T normal human fibroblasts; lane b, NIH/3T3 cells; lanes c and d, T24 bladder carcinoma cell line and T24 NIH/3T3 transfectants; lanes e and f, HS578T mammary carcinosarcoma cell line and HS578T NIH/3T3 transfectants; lanes g and h, Calu-6 lung carcinoma and Calu-6 NIH/3T3 transfectants; lanes i and j, A427 lung carcinoma and A427 NIH/3T3 transfectants; lanes k and l, SW1271 lung carcinoma and SW1271 NIH/3T3 transfectants; and lanes m and n, Hs242 lung carcinoma and Hs242 NIH/3T3 transfectants. Positions and sizes (in kDa) of molecular weight marker proteins and the region containing the p21s are indicated on the right.

The p21 molecules encoded by various human *ras* oncogenes also differed in their electrophoretic mobilities (Fig. 1). This variability appeared to be independent of both the type of *ras* oncogene present and its level of expression. However, the altered mobilities of the p21 products were stable genetic properties, since in each case similar patterns were observed upon oncogene transmission to NIH/3T3 cells (Fig. 1, lanes d, f, h, j, l, and n). Since the levels of p21 were generally higher in NIH/3T3 transfectants as compared to the parental tumor-cell lines, we utilized the transfectants in subsequent studies.

p21 Proteins Encoded by Human *ras* Oncogenes Are Not Detectably Phosphorylated. To determine whether the heterogeneity observed in the mobilities of different p21 molecules might be accounted for by differences in phosphorylation, NIH/3T3 transfectants containing representative H-, K-, and N-*ras* oncogenes were metabolically labeled with [^{32}P]orthophosphate and the cell extracts were analyzed by immunoprecipitation using monoclonal antibodies Y13-259 and YA6-172 (Fig. 2). In contrast to the readily observed phosphorylation of p21 expressed by Harvey-MSV-transformed NIH/3T3 cells (lane a), the p21 proteins encoded by the human *ras* oncogenes (Fig. 2, lanes b–f) were not detectably phosphorylated. These results suggested that differences in phosphorylation were not responsible for differences in p21 electrophoretic mobility.

Characteristic Altered Electrophoretic Mobilities of p21 Proteins with Position-12 or -61 Substitutions. The altered p21 molecules could be classified as slow- and fast-moving species when compared with mouse endogenous p21 bands (Fig. 3). H-*ras*-derived T24 (lane 2) or HS578T (lane 3) oncogenes, which had suffered mutations in their 12th codons resulting in substitution of valine or aspartic acid, respectively, for glycine (Table 1), yielded p21s that migrated as slow-moving species. In contrast, H-*ras*-derived Hs242 and JPT26 (lanes 4 and 5) as well as N-*ras*-derived SW1271 (lane 6) oncogenes, which were activated by mutations in their 61st codons (Table 1), yielded fast-moving species (Fig. 3). Proteins with low mobilities were observed in NIH/3T3 transformants of K-*ras* oncogenes of the Calu-6 (lane 7) and A427 (lane 8) human tumor lines. Although the mutations affecting these oncogenes have not been characterized, the mobilities of these p21 proteins suggest lesion(s) affecting the 12th codon.

Effect of Activating Lesions at Both Position 12 and 61 on p21 Mobility. Using the strategy diagrammed in Fig. 4*A*, we constructed a hybrid gene between Hs242 and T24 oncogenes to assess the combined effect of both molecular le-

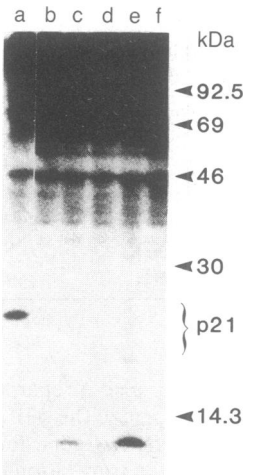

FIG. 2. Human *ras*-oncogene-encoded p21s are not detectably phosphorylated. NIH/3T3 cells transformed by Harvey-MSV (lane a), or by T24 (lane b), Hs242 (lane c), SW1271 (lane d), Calu-6 (lane e), or A427 (lane f) oncogenes were labeled with [^{32}P]orthophosphate. After immunoprecipitation with monoclonal antibody Y13-259, p21 proteins were analyzed by NaDodSO$_4$/PAGE and autoradiography.

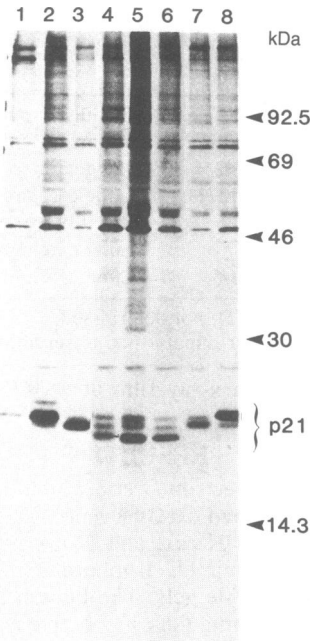

FIG. 3. Characteristic p21 mobilities are associated with mutations in 12th or 61st codons. Control NIH/3T3 cells (lane 1) and NIH/3T3 cells transformed by T24 (lane 2), HS578T (lane 3), Hs242 (lane 4), JPT26 (lane 5), SW1271 (lane 6), Calu-6 (lane 7), or A427 (lane 8) oncogenes were labeled with [^{35}S]methionine. After immunoprecipitation with monoclonal antibody Y13-259, p21 electrophoretic mobilities were compared by NaDodSO$_4$/12% PAGE and fluorography.

sions on *ras* transforming-gene function and p21 mobility. A T24/Hs242 recombinant possessing mutations at both codon 12 (glycine→valine) and codon 61 (glutamine→leucine) transformed NIH/3T3 cells with an efficiency similar to that of either the Hs242 or the T24 oncogene (Fig. 4*A*). These results established that the two mutations together were not antagonistic to the transforming properties of the altered p21 molecule. The p21 protein encoded by the Hs242/T24 recombinant had an electrophoretic mobility between those of the fast-moving Hs242 (lane 5) and slow-moving T24 (lane 4) oncogene-encoded p21 molecules (Fig. 4*B*). These findings indicate that the two activating lesions had apparently equivalent, opposing effects on the conformation of the p21 molecule as measured by electrophoretic mobility under our assay conditions.

Processing of p21 Proteins Having Different Mutations. Previous studies with Harvey-MSV p21 have shown that, after a 1-hr pulse-label, the radioactive protein appears mostly as pro-p21, which is converted during a 4-hr chase to a processed form of greater mobility. After 24 hr of chase, there appears a phosphorylated form that migrates more slowly than pro-p21 (40).

We observed that the processing of human *ras*-oncogene-encoded p21s was very rapid. In fact, after 5 min of exposure of the cells to [^{35}S]methionine, processing of the pro-p21 was >50% complete within 15 min and essentially complete by 2 hr (Fig. 5). No forms other than the processed p21 molecule were detected even after 24 hr (data not shown). Fig. 5 shows that the mobility of the Hs242-encoded p21 precursor

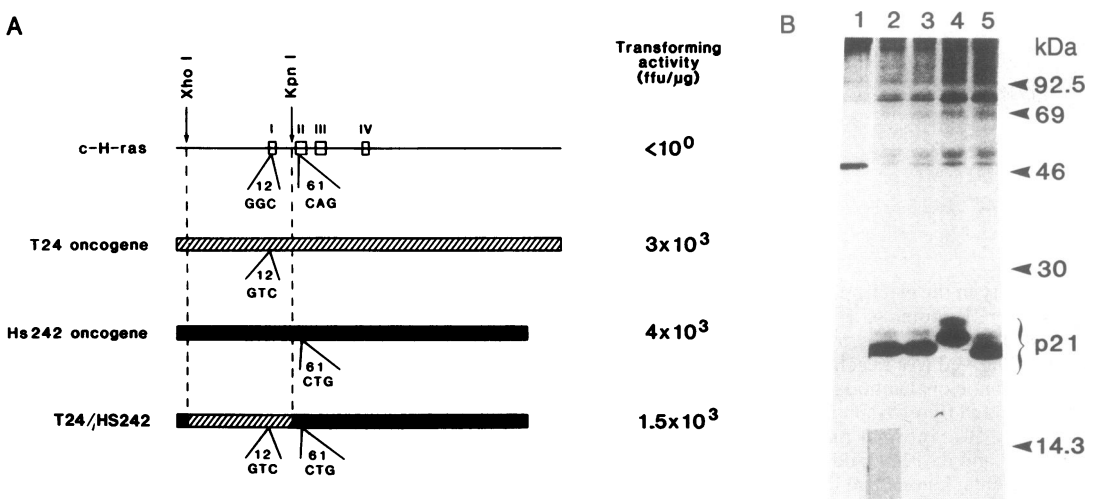

FIG. 4. Transforming potential of and p21 protein expression by a recombinant of the T24 and Hs242 oncogenes. (*A*) A hybrid gene was constructed between the T24 (14) and Hs242 oncogenes (32) by substituting the *Kpn* I/*Xho* I fragment of the T24 oncogene for the *Kpn* I/*Xho* I fragment of the Hs242 oncogene. Thus, the resulting plasmid possessed mutations in both the 12th (glycine→valine) and 61st (glutamine→leucine) codons. I–IV are exons of the c-H-*ras* gene. Transforming activities of various DNA recombinant clones in the NIH/3T3 transfection assay are expressed as focus-forming units (ffu) per μg of DNA. (*B*) Comparison of the electrophoretic mobilities of p21 proteins encoded by the T24/Hs242 hybrid gene (lanes 2 and 3) and the T24 (lane 4) and Hs242 (lane 5) oncogenes by immunoprecipitation and NaDodSO$_4$/PAGE. The p21 protein expressed by control NIH/3T3 cells (lane 1) was used as a marker of normal p21 electrophoretic mobility.

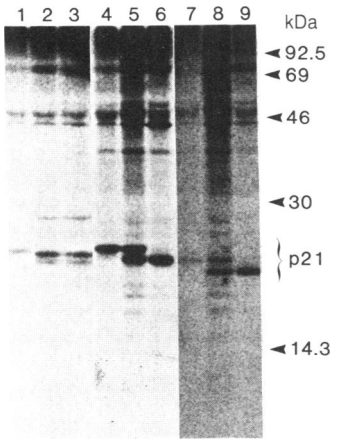

FIG. 5. Posttranslational processing patterns of p21 proteins encoded by T24 and Hs242 oncogenes. Transfectants containing T24 or Hs242 oncogenes were labeled with [^{35}S]methionine (750 μCi/ml of medium) for 5 min. After incubation for 15 min or 2 hr in medium containing 1% (wt/vol) unlabeled methionine, the p21 proteins were immunoprecipitated with monoclonal antibody YA6-172 and analyzed by NaDodSO$_4$/14% PAGE followed by fluorography. NIH/3T3 cells analyzed after 0.5 min, 15 min, or 2 hr of chase (lanes 1–3); T24 oncogene transfectant (lanes 4–6) and Hs242 oncogene transfectant (lanes 7–9) analyzed at same time points.

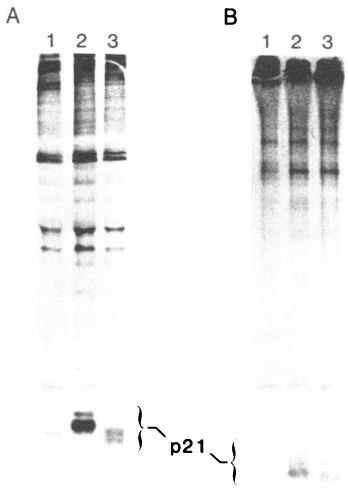

FIG. 6. Analysis of p21 proteins under reducing and nonreducing conditions. NIH/3T3 cells transformed by T24 or Hs242 oncogenes were labeled with [^{35}S]methionine, and immunoprecipitates obtained with monoclonal antibody Y13-259 were analyzed by NaDodSO$_4$/PAGE in the presence (*A*) or absence (*B*) of 2-mercaptoethanol. Lanes 1, NIH/3T3 cells; lanes 2, NIH/3T3 cells transformed by the T24 oncogene; lanes 3, NIH/3T3 cells transformed by the Hs242 oncogene. The electrophoretic mobilities of p21 proteins under reducing and nonreducing conditions were determined relative to lysozyme (14.3 kDa), carbonic anhydrase (30 kDa), and ovalbumin (46 kDa).

(lane 7) was greater than that of the T24-encoded p21 precursor (lane 4). Moreover, the differences in relative mobilities of precursors (pro-p21) and products (p21) appeared to be similar, suggesting the absence of any obvious difference in the effect of position-12 and -61 activating lesions on processing of the p21 product. These findings are consistent with observations that p21 processing, whether due to cleavage (40) or to posttranslational addition of a lipid moiety (41), occurs at a site distant from the 12th and 61st amino acids, near the carboxyl terminus of the molecule.

Role of Cysteine Residues in Maintaining p21 Protein Structure. Amino acid sequence analysis of p21 molecules encoded by different *ras* genes has revealed the presence of four highly conserved cysteine residues (42). It is well established that native protein conformation can be affected by either intra- or intermolecular disulfide linkages. Thus, we compared the mobilities of p21 molecules in NaDodSO$_4$/PAGE in the presence and absence of reducing agent. As shown in Fig. 6, striking differences were observed. In contrast to their sharply defined mobilities under reducing conditions (Fig. 6*A*, lanes 1–3), p21 molecules exhibited much higher mobilities as well as a more diffuse banding pattern under nonreducing conditions (Fig. 6*B*, lanes 1–3). We stress that under nonreducing conditions we observed little, if any, difference between the mobilities of gene products altered at position 12 or 61.

To confirm that the proteins identified with monoclonal antibody Y13-259 were p21 species, we carried out competition studies utilizing bacterially expressed and partially purified BALB MSV p21 (unpublished data). When the antiserum was preincubated with this protein, immunoprecipitation of p21 species identified under both reducing and nonreducing conditions (Fig. 6) was completely inhibited (data not shown).

We observed that the amount of p21 detected under nonreducing conditions was generally lower than that observed in the presence of reducing agents. This may be explained in part by the more diffuse banding pattern of the protein under nonreducing conditions. However, it is also possible that, in their nonreduced forms, p21 proteins may be complexed with other cellular proteins. However, we did not detect any major discrete high molecular weight bands that contained specific p21 reactivity.

DISCUSSION

The *ras* oncogenes have been detected in a wide array of human malignancies. It is well established that these transforming genes are most commonly activated by single-nucleotide substitutions at one of two major "hot spots" in their coding sequences. Despite the likely importance of these altered gene products in the neoplastic process, little is known about the structure and function of *ras*-encoded p21 proteins except that they bind guanine nucleotides (26, 43) and some are phosphorylated *in vivo* (23). In this report, we compared different human *ras* oncogene products with their normal counterparts in an effort to elucidate native p21 structure and the effects of the major activating lesions on conformation of the p21 molecule.

We found that human *ras*-encoded p21 molecules exhibited strikingly different electrophoretic mobilities in NaDodSO$_4$/PAGE in the presence and absence of reducing agents. The proteins yielded discrete, sharply defined bands under reducing conditions but gave more diffuse bands of higher mobility in the absence of reducing agent. These findings argue strongly for the presence of disulfide linkages in the native form of the protein. Previous studies have documented that proteins containing intermolecular disulfide bonds generally migrate more slowly under nonreducing conditions (44–46). In contrast, β-lactamase, a protein with a single intramolecular disulfide bond, has been shown to migrate faster in NaDodSO$_4$/PAGE in its nonreduced form (47). Thus, the more rapid migration of p21 under nonreducing conditions suggests the existence of intramolecular disulfide bonds as well as a compact conformation of the native p21 molecule.

We observed differences in mobilities of *ras* oncogene products that were only unmasked by NaDodSO$_4$/PAGE under reducing conditions. Although the altered migration of p21 proteins under these conditions has previously been reported (15, 28, 32), we were able to ascribe a pattern of consistent alterations to the effects of position-12 or -61 activating lesions. While most activated genes analyzed were H-*ras*

alleles, we observed altered mobilities of the p21 translation products of activated N-*ras* and K-*ras* genes consistent with the patterns observed for H-*ras* gene products. Analysis of additional N-*ras*, as well as K-*ras*, oncogenes with defined codon-12 or -61 mutations has further substantiated these conclusions (unpublished observations). However, whether all activating lesions at these sites lead to altered electrophoretic mobilities of *ras* translational products is not known.

Whereas position-12-activated *ras* proteins migrated with lower mobilities than that of the normal, position-61-activated *ras* proteins invariably showed higher mobilities. That these alterations induced similar but opposing effects on p21 mobility was established by analysis of a p21 recombinant molecule containing both activating lesions. This p21 protein was of intermediate electrophoretic mobility, close to that of the normal. Our findings that the recombinant transforming gene exhibited transforming activity comparable to that of either singly activated *ras* oncogene established that the two lesions did not negatively complement one another with respect to transforming-gene function.

Our ability to discriminate effects of position-12 and -61 lesions on protein migration only under reducing conditions suggests that, in the native p21 molecule, regions containing these lesions are either not exposed or under conformational constraints due to intramolecular disulfide bonds. There are four conserved cysteines within the p21s of all known members of the *ras* gene family (42). Site-specific mutagenesis of these residues should help to resolve which are involved in disulfide bonds critical to the native conformation of the molecule and determine the effects of alteration of these residues on p21 function.

Our findings offer a possible application to the rapid diagnosis of *ras* oncogenes in tumors. The different mobilities under reducing conditions of position-12- and position-61-activated *ras* gene products should be readily detectable in immunoblots of tumor tissues. In view of the inherent insensitivity and time-consuming nature of the transfection assay for *ras* oncogenes, such an approach could help to provide a better understanding of the distribution and frequency of *ras* oncogenes in human malignancies.

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