Journal of Cellular Biochemistry 80:483–490 (2001)

FAST TRACKS

# Molecular Interaction Between Human Tumor Marker Protein p150, the Largest Subunit of eIF3, and Intermediate Filament Protein K7

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**Abstract** The human tumor marker protein p150 was identified as the largest subunit of eukaryotic translation initiation factor 3 (eIF3) (also known as p170/p180). Its expression level is not only upregulated in many transformed cell lines, but also in several human cancers including breast, cervical, esophageal, and stomach carcinomas. The function of p150 in cancer and initiation of translation are not well understood. Using the yeast two-hybrid genetic screen, we found that a portion of p150 interacts with hPrt1, another subunit of eIF3, and cytokeratin 7, an intermediate filament protein. The interactions between p150 and hPrt1, and between p150 and cytokeratin 7 were verified both in vivo and in vitro. The interaction site for hPrt1 was mapped to the carboxyl half of the coiled-coil region of the p150 protein between amino acids 664–835. The expression of hPrt1 was clearly upregulated in cancer tissue, similarly to that of p150. By contrast, no substantial difference in the expression level of cytokeratin 7 was observed between cancer and normal breast tissue, suggesting that cytokeratin 7 expression is not co-regulated with p150. Taken together, our studies suggest a new role for p150 in translation initiation, possibly by acting as an adapter molecule between the translation initiation apparatus and the cytoskeleton structure in the cell. J. Cell. Biochem. 80:483–490, 2001. © 2001 Wiley-Liss, Inc.

**Key words:** eIF3; cytokeratin k7; p150/p180; tanslation initiation factor; breast cancer

A novel mouse protein with an estimated molecular weight of 150 kDa was found to be highly expressed in transformed cell lines. The protein was partially purified and the cDNA cloned [Bachmann et al., 1997]. Sequence alignments indicated that it is the murine homologue of p180, the largest subunit of human eukaryotic translation initiation factor 3 (eIF3) [Johnson and Merrick, 1997] and of p110, its yeast counterpart [Vornlocher et al., 1999]. Database mining identified homologous sequences from *C. elegans, N. tabacum, D. melanogaster, A. thaliana, and Z. mays*, which are likely to be the corresponding eIF3 subunits in these organisms. The human homologue was found to be overexpressed in mammary carcinoma when compared with normal breast tissue taken from the same individual [Bachmann et al., 1997]. Additional analysis in cervical [Dellas et al., 1998] and esophageal carcinomas [Chen and Burger, 1999] has shown a correlation between high p150 expression, well-differentiated cancers, and a better prognosis for the patient. More recently, studies in gastric carcinomas indicated a close relationship between p150 expression and tumor cell apoptosis [Chen and Burger, submitted for publication].

In eukaryotes, at least 10 translation initiation factors have been implicated in the initiation step of protein synthesis. Among them, eIF3 is the largest and the most complex, with a size of ~650 kDa. cDNAs encoding at least 10 subunits of eIF3 have been cloned [Vornlocher et al., 1999]. eIF3 has been implicated in a variety of roles during initiation of translation. First, it is thought to be an anti-association factor binding to the 40S subunit, thereby preventing the re-association of the 60S to the 40S subunit prior to formation of the 43S preinitiation complex [Merrick and Hershey, 1996]. Second, eIF3 prevents dissociation of the Met-tRNA$_i$, eIF2, and the GTP ternary

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Received 29 September 2000; Accepted 2 October 2000

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This article published online in Wiley InterScience, December XX, 2000.

complex caused by the addition of RNA [Gupta et al., 1990]. Lastly, it is required for mRNA binding [Benne and Hershey, 1978; Trachsel and Staehelin, 1979], probably via interaction with the cap-binding protein complex eIF4F [Lamphear et al., 1995].

The role of p150 in translational control and in cancer development remains unclear. The yeast homologue of p150 is encoded by the *TIF*32 gene, which has been shown to be essential [Vornlocher et al., 1999]. The human homologue of p150 interacts with several other initiation factors including eIF3 p116 (hPRT1) [Méthot et al., 1997], eIF3 p44 [Block et al., 1998], and eIF4B [Méthot et al., 1996]. Limited biochemical evidence suggest that p150 might play a central role in eIF3 function by modulating multiple protein–protein interactions [Méthot et al., 1997].

The primary sequence of p150 suggests several interesting features. Among the potential homologues in other organisms, the N-terminal part of the sequence (amino acids 1–328 in p150) shows stronger homology (e.g., 35% identity with yeast p110) than the rest of the protein (20% identity with the yeast homologue). The middle region, covering amino acids 552–835 in p150, is predicted to form a coiled-coil structure according to the method described by Lupas et al. [1991] and the CoilScan program from the GCG (Genetics Computer Group), Wisconsin Package. This feature is shared by all of the homologues in this group. The coiled-coil structure/heptad repeat is characterized by a bundle of $\alpha$-helices that winds into a superhelix. This structure has been found in many proteins including myosin and several transcriptional activators, where it mediates dimerization and oligomerization [reviewed by Lupas, 1996]. Finally, as pointed out by Bachmann et al. [1997] and Johnson and Merrick [1997], the carboxyl terminal domain of p150 (amino acid 925–1172) is highly charged and contains some unusual repetitive sequences. The length of this domain varies significantly among different p150 homologues. Those from the more distant organisms such as yeast do not contain this domain. It is possible that this domain might be important in modulating specific interactions with other proteins or RNA molecules in higher eukaryotes.

The purpose of this study was to add to our understanding of the biological function of p150 and of its role in cancer development. As a first step toward this goal, we decided to search for cellular proteins that interact with p150. The N-terminal domain and the coiled-coil region of p150 were fused to a GAL4 DNA binding domain and used as baits for the yeast two-hybrid screening. These two domains were chosen not only because they are present in all of the p150 homologues, but also because they are almost identical to their human counterpart p180. N328 has two conserved amino acid changes, while M282 has one threonine-to-asparagine variation at position 3, and two other conserved changes. Therefore the information obtained from the screening can be correlated directly with human cancer cells. Each bait plasmid was co-transformed with a HeLa cell cDNA library fused to a GAL4 activation domain. Interestingly, we found that p150 not only interacts with hPrt1, another subunit of eIF3 as one might expect, it also interacts with an intermediate filament protein, cytokeratin 7 (K7). Moreover, we found that the expression level of hPrt1, but not K7, is upregulated in cancer tissues, similar to that of p150.

## MATERIALS AND METHODS

### Yeast Two-Hybrid System

Coding sequences comprising the appropriate nucleotide residues were isolated by PCR and cloned in-frame with the GAL4 DNA binding domain into plasmid pAS2-1 (Clontech). The resulting plasmids were designated as pASN328 for the N-terminal domain (amino acid 1–328), and pASM282 for the coiled-coil region (amino acids 552–835). The latter was also divided into two smaller baits and subcloned as pASM116N, and pASM165C. Each of the plasmids was co-transformed with $3 \times 10^6$ cDNAs from a human HeLa MATCHMAKER cDNA library (Clontech) into yeast strain Y190. The transformants were plated onto a selective medium. Colonies were screened for *LacZ* activity using the freeze–thawing procedure. Putative interacting clones were retested by yeast mating as described by the manufacturer. Confirmed positive clones were then sequenced and the resulting sequences were used to scan the GenBank and EMBL databases for homologous matches using the BLAST search program. Full length cDNAs for cytokeratin 8 and vimentin were isolated using the Expand$^{TM}$ Long Template PCR System (Roche Molecular

Biochemicals) performed on HeLa marathon ready cDNA (Clontech) or human brain cDNA library (kindly provided by Dr. Xavi Fernanderz, Friedrich Miescher Institute).

### Fusion Proteins and Antibodies

GST fusion protein constructs were prepared by subcloning the desired nucleotide regions of p150, hPrt1, and Cytokeratin 7 into the *Eco*RI and *Sal*I sites of pGEX-4T3 (Amersham-Pharmacia). Fusion proteins were expressed in *E. coli* and purified by glutathione-Sepharose (Amersham-Pharmacia) following the manufacturer's instructions. Polyclonal antibodies from rabbits were produced and purified according to standard procedures, using purified fusion proteins as antigens [Harlow and Lane, 1988]. The cytokeratin 7 mAb was from Sigma. Dr. J. W. B. Hershey, University of California at Davis kindly provided the goat anti-eIF3 antibody.

### Affinity Chromatography

Affinity columns were prepared by immobilizing GST fusion proteins onto the glutathione-Sepharose beads as described by the manufacturer. Four milligrams of HeLa cytosolic extract were loaded onto a 50 μl affinity column containing the appropriate GST fusion protein. The beads were washed with PBS in the presence of 0.5% Tween and the bound protein was eluted with SDS sample buffer (50 mM Tris–Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.1% bromophenol blue).

### Co-immunoprecipitation Assay

HeLa cells were solubilized with ice-cold lysis buffer as described [Harlow and Lane, 1988]. The lysate was centrifuged at 10,000*g* for 10 min at 4°C. Proteins (1.5 mg) were mixed with rabbit anti-27K antibody that was cross-linked to protein A-Sepharose (Pharmacia), and incubated for 2 h at 4°C. As a control pre-immune rabbit serum beads were prepared and used in the assay. The immunocomplexes were washed with 50 mm Tris–Cl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 and collected in SDS sample buffer.

### Tumor and Normal Tissue Extracts and Immunoblotting

Tissue extraction and Western blotting were carried out as previously described [Bachmann et al., 1997].

## RESULTS

### The Coiled-Coil Region of p150 Interacts Specifically With Two Proteins in the Yeast Two-Hybrid Assay

Initial screening using pASN328 did not produce any true positive clones and was not pursued further. Screening using pASM282 was more fruitful. Figure 1a shows a schematic diagram of the bait construction, and Figure 1b shows the coiled-coil prediction of p150 using the GCG package. As shown in the figure, the major part of M282 fragment is predicted to form a coiled-coil structure. From approximately 2 million transformants, 19 clones were isolated that interacted specifically with the coiled-coil region of p150. Seven of these clones were found to encode the partial cDNA for protein hPrt1, the second largest subunit of mammalian eIF3 [Méthot et al., 1997]. The other 12 clones encoded the partial cDNA sequence of cytokeratin polypeptide 7 (K7), an intermediate filament protein [Glass and Fuchs, 1988]. Table I summarizes the levels of β-galactosidase activity obtained from four different experiments. The coiled-coil fragment M282 interacted with both $hPrt1_{129-873}$ and $K7_{325-468}$, generating β-galactosidase activities of 19 muller units for $hPrt1_{129-873}$, and 47 muller units for $K7_{325-468}$. A positive control in which murine p53 interacts with SV4O large T-antigen (provided by the manufacturer) gave 188 muller units.

To determine whether the entire coiled-coil region is needed for this interaction, two smaller bait constructs were generated which encompass the N-terminal 116 amino acids (pASM116N) and C-terminal 165 residues (pASM165C), and were tested for their ability to interact with hPrt1 and K7. In the presence of either pGAD-$hPrt1_{129-873}$ or pGAD-$K7_{325-468}$, pASM116N failed to support the growth of yeast strain Y190 on selective media lacking Trp, Leu, and His. This result indicates that the N-terminal region of M282 does not contribute directly to the specific interaction between p150 and hPrt1, or between p150 and K7. The C-terminal portion of M282, pASM165C, supported the growth on selective media in the presence of pGAD-$hPrt1_{129-873}$ and pGAD-$K7_{325-468}$ although to a lesser extent. Interaction between M165C and $hPrt1_{129-873}$ produced a β-galactosidase activity of 11 muller units, slightly lower than the full length M282. The same C-terminal frag-

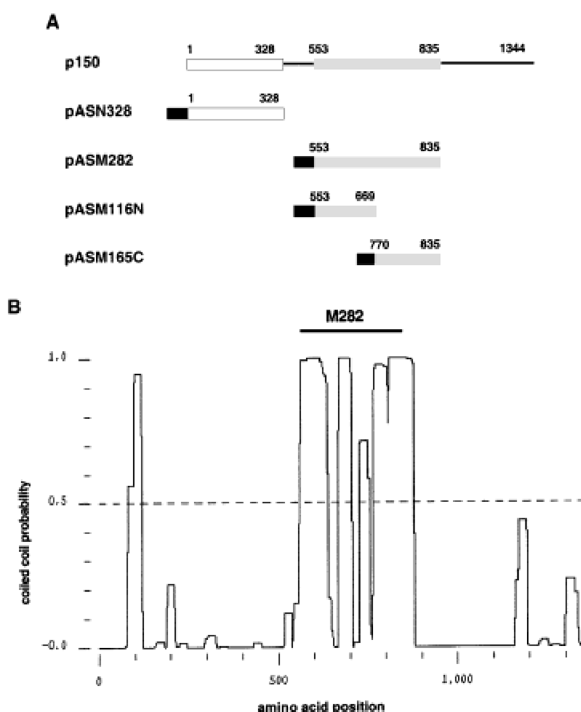


**Fig. 1.** (**A**) Schematic representation of p150 and domains used as baits in the yeast two-hybrid screening. The fragments were cloned in-frame with Gal4 DNA binding domain. Filled area: Ga14 DNA binding domain. Empty area: N-terminal domain of p150; Shaded: the coiled-coil region of p150. (**B**) Coiled-coil prediction of p150 generated using the program CoilScan from the UWGCG package. Fragment M282 spans the middle region that shows 100% probability of forming a coiled-coil structure.

ment however, only interacted weakly with $K7_{325–468}$ as judged by the slow growth rate on selective media. Because of this slow growth, we were unable to quantitate the β-galactosidase activity in our *lac*Z assay. This result raises the possibility that the N-terminal portion of M282 might be needed to increase the stability of the C-terminal region.

## The Coiled-Coil Region of p150 Does not Form Homodimers

It is known that some coiled-coil proteins can form dimer or multimer complexes [Lupas, 1996]. In order to test if p150 can self-associate, the same coiled-coil region of p150 was subcloned into plasmid pGAD-GH as a fusion of Ga14 activation domain. As shown in Table I, column 3, the dimer interaction was undetectable. Moreover, we did not obtain any true positive clones that encode p150 in our yeast two-hybrid screening, further supporting our observation that p150 uses this region to interact with proteins other than itself.

## p150 Interacts with hPrt1 and Cytokeratin 7 Specifically Both In Vitro and In Vivo

We performed an in vitro binding assay using bacterially produced GST-$hPrt1_{129–873}$ and GST-$K7_{325–468}$ immobilized on glutathione-Sepharose columns. A GST protein immobilized on glutathione-Sepharose was used as a control in parallel. Equal amounts of HeLa cell extracts were loaded onto the fusion protein and control columns and bound proteins were eluted with SDS sample buffer and analyzed by Western blot analysis. As shown in Figure 2a, p150 was detected in the eluates from both GST-$hPrt1_{129–873}$ and GST-$K7_{325–468}$ columns (lanes 3 and 5 from the left) but not from the GST control columns (lanes 2 and 4 from the left), indicating that the interactions between p150 and $hPrt1_{129-873}$, and between p150 and $K7_{325–468}$ are specific.

To verify that this interaction exists with the full-length proteins in vivo, HeLa cell extracts were immunoprecipitated by polyclonal antibodies directed against hPrt1 and K7. As

**TABLE I. Quantitative β-Galactosidase Assay of p150 With its Interaction Partners**

| | Gal4-AD vector | | | | | | |
|---|---|---|---|---|---|---|---|
| | pGAD-$hPrt1_{129–873}$ | | pGAD-$K7_{325–468}$ | | pGAD-M282 | | pTDi-1 |
| Gal4-BD vector | *His*$^+$ | *LacZ* | *His*$^+$ | *LacZ* | *His*$^+$ | *LacZ* | *LacZ* |
| pASM282 | + | 18.5 ± 2.0[a] | + | 47.0 ± 11.8 | – | ND | ND |
| pASM116N | – | ND | – | ND | ND | ND | ND |
| pASM165C | + | 11.3 ± 1.7 | + | ND | ND | ND | ND |
| pVA3-1 | ND | ND | ND | ND | ND | ND | 188.4 ± 11.4 |

[a]The results are averages of four independent experiments. The number is shown in muller unit, which is defined as the enzyme amount required to produce 1 μmol of *o*-nitrophenol per minute.

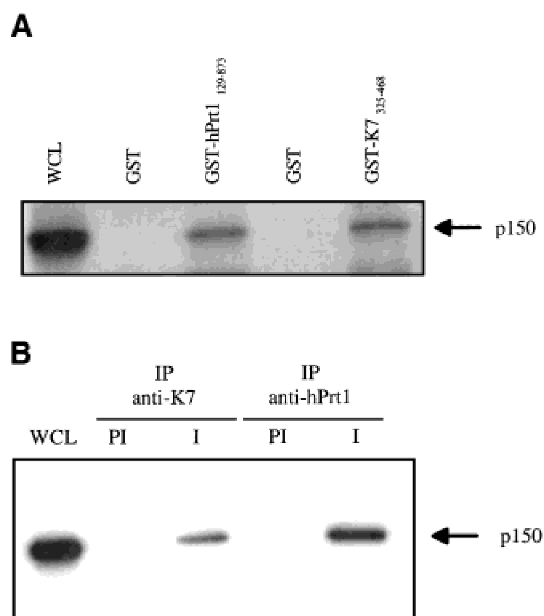


**Fig. 2.** (**A**) Affinity pull-down of p150 on immobilized GST-$K7_{325–468}$ and GST-$hPrt1_{129–873}$. Western blot analysis with a chicken anti-mouse p150 antibody of input HeLa cell lysate (**lane 1** on the left) and the eluates from the indicated GST fusion columns. The p150 band is labeled as shown. (**B**) Interaction of p150 with K7 and hPrt1 in HeLa cells. Western blot analysis with the same anti-mouse p150 antibody of HeLa whole-cell lysate (WCL) (**lane 1** on the left), and the immune complexes precipitated by preimmune serum for K7 (**lane 2**), anti-K7 antibody (**lane 3**), preimmune serum for hPrt1 (**lane 4**), and anti-hPrt1 antibody (**lane 5**).

shown in Figure 2b, p150 was found only in the immunoprecipitates with antigen affinity-purified anti-K7 and anti-hPrt1 (lanes 3 and 5 from the left), but not in the immunoprecipitates obtained with preimmune sera (lanes 2 and 4 from the left). Crude antibodies gave similar results (data not shown).

### hPrt1, but not K7, is Upregulated in Human Mammary Carcinoma

p150 was initially discovered as a tumor antigen whose expression level is upregulated in human mammary carcinoma [Bachmann et al., 1997]. We therefore investigated whether the expression level of the two interacting partners is co-regulated with p150. Human primary breast cancer tissues from four patients were chosen at random from the Institute of Pathology at the University of Basel. Western blot analysis with specific antibodies was used to analyze the expression levels of p150, hPrt1, eIF3, and K7 (Fig. 3). Normal tissue from the same breast was used as an internal control in each case. All four patients showed a higher level expression of p150 in tumors than in the normal tissue (Fig. 3, Panel a), which is consistent with a previous analysis [Bachmann et al., 1997].

We then investigated the expression level of hPrt1 using affinity-purified anti-Prt1 antibody. Panel b shows that in all four cases, hPrt1 is upregulated in the tumor samples but not in the normal tissues. This overexpression can be verified using antibodies against the whole eIF3 complex (kindly provided by Dr. J. Hershey, University of California at Davis). As shown in Panel c, overexpression of both p150 and hPrt1 can be seen clearly in the tumor tissue lanes. However, under our blotting condition, we were unable to detect the expression of other subunits in the eIF3 complex. This might be due to the lower titer against these

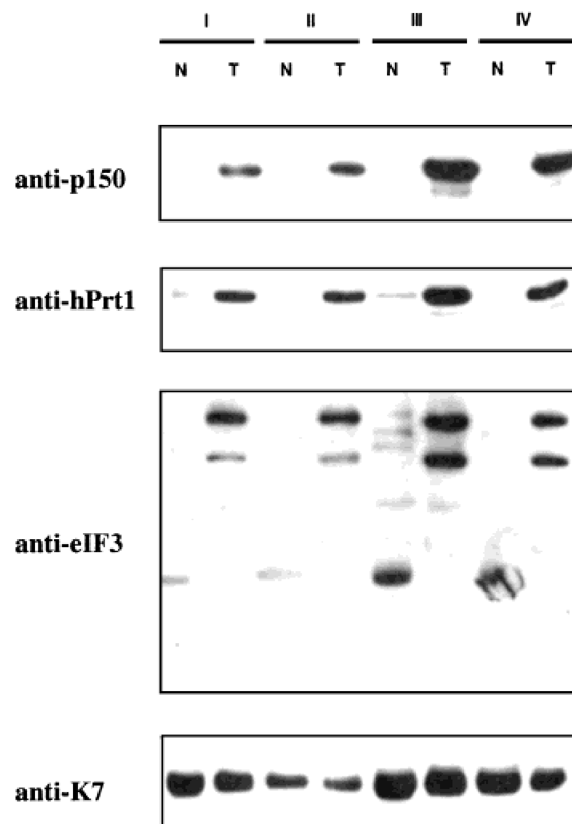


**Fig. 3.** Western blot analysis of human breast tissues from four randomly chosen patients. N: normal tissue; T: tumor tissue. The blot is probed with the same anti-p150 antibody used in Figure 2 (Panel 1 from the top); rabbit anti-hPrt1 antibody (Panel 2); goat anti-eIF3 antibodies (Panel 3); and monoclonal antibody for K7 (Panel 4). The corresponding protein bands are indicated on the right.

proteins present in the antibody preparation. Alternatively, these subunits might not have their expression co-regulated with the other two subunits. The simultaneous increase in the expression level of p150 and hPrt1 is consistent with the observation that the expression levels of their yeast counterparts (viz p110 and p90) are also coupled [Vornlocher et al., 1999].

Analysis using monoclonal antibody against cytokeratin 7 showed a clear contrast with hPrt1 (Panel d). The expression level of K7 varied slightly between tumor and normal tissues but no clear trend could be observed in all cases, indicating that the expression of p150 and K7 is not coupled.

## DISCUSSION

It has been suggested for a number of years that mRNA and protein translational machinery is associated with the cytoskeleton framework of the cell. Electron microscopy, sucrose gradient, and immunohistochemical analysis have shown that the cell matrix fraction from a variety of cell lines contains polysomes, translation initiation factors including eIF3, and approximately 70% of the cellular mRNA [Cervera et al., 1981; Howe and Hershey, 1984; Lenk et al., 1977; Zumbé et al., 1982]. The biological significance of the association of protein synthesis with the cytoskeleton network of the cell remains unclear. It has been proposed that this association serves as the basis of a mechanism for the targeting of mRNAs and the compartmentalization of protein synthesis [Hesketh, 1994], which seems crucial in terms of developmental determinants during morphogenesis, spatial control of macromolecular assemblies, isoform segregation and development of cell polarity [Singer, 1992; Takizawa et al., 1997].

Our present study points to a new role for intermediate filament proteins (IFs). We demonstrate for the first time a direct physical interaction between a subunit of eIF3 and the intermediate filament protein cytokeratin 7, in contrast to earlier studies that suggest an association of polysomes or mRNAs with the actin containing filaments and microtubules [Hesketh, 1994; Bassell and Singer, 1997]. Cytokeratins are part of the intermediate filament protein family. The major role of intermediate filaments is to provide essential structural support for maintaining cellular integrity and the mechanical properties of cytoplasm [see review, Chou et al., 1997]. All intermediate filament proteins share a common structural feature which includes an N-terminal "head" segment, a C-terminal "tail" segment, and a highly conserved, $\alpha$-helical central rod domain (coiled-coil). Interestingly, this structural arrangement is also shared by p150 (see Fig. 1b). How specific is this interaction between K7 and p150 among other IFs? We have tested the potential interaction between p150 and K8, a closely related cytokeratin from the same cell type; and between p150 and vimentin, a mesenchymal IF. Using yeast two-hybrid, we found that none of these two IF proteins interacts with the coiled-coil region of p150 (L. Lin, unpublished observation). The K7 fragment that we isolated by the yeast two-hybrid method contains a portion of the highly conserved rod domain plus the tail segment. This result therefore suggests that the C-terminal tail sequence of K7 might contain additional information that allows it to interact with p150 specifically.

Previous analysis identified the region between amino acids 147–255 of hPrt1 as its interaction site with the human p150 [Méthot et al., 1997]. This region contains a putative RNA recognition motif (RRM) and it has been speculated that its RNA binding activity and specificity are modulated by human p150. The site of interaction with hPrt1 on p150 lies between amino acids 669–835, the carboxyl half of the coiled-coil region. It is easy to imagine that K7 and p150 interact via their coiled-coil domains. The mode of interaction of hPrt1 with p150 is not apparent and will have to wait for a more detailed structural analysis.

The results presented here do not address the mechanism by which p150 expression is increased in in vitro transformed cells, dedifferentiated cells, and tumors [Bachmann et al., 1997]. In particular, it is not clear how this increase relates to the interaction of p150 with IF or other subunits of eIF3. There have been several cases in which overexpression of translational components correlates with the rate of cell growth and proliferation. Several initiation and elongation factors were found to be either highly expressed in cancer cells [Edmonds et al., 1996; Nupponen et al., 1999] or to cause malignant transformation when overexpressed in NIH 3T3 cells [Donzé et al., 1995; Fukuchi-Shimogori et al., 1997]. It is likely that

initiation factors have additional functions other than initiation of protein synthesis. For example, yeast PRT1 was originally identified as a cell division cycle (cdc) mutant [Hartwell and McLaughlin, 1968] and might play a dual role in proliferation control and translation initiation [Hanic-Joyce et al., 1987].

The molecular interaction between p150 and K7 suggests a new role for p150, in which p150 functions as an adapter molecule between the eIF3 complex and the intermediate filament bundle. In our GST pull down experiment using GST-$K7_{325–468}$, we detected the presence of not only p150 (Fig. 2a) but at least one other subunit of eIF3, namely hPrt1 [L. Lin, unpublished observation]. This result supports the idea that p150 serves as a bridging molecule between eIF3 and K7. Earlier evidence suggests that p150 might not be crucial for eIF3 function [Chaudhuri et al., 1997] although it is an integral part of the mammalian eIF3 [Asano et al., 1998; Valasek et al., 1998]. Together, these results imply a structural as well as a functional role for the largest subunit of eIF3. The overexpression of p150 in tumor cells might be the consequence of a series of signaling events, perhaps through another subunit of eIF3, or other translational components, or even K7 itself. The expression level of K7 was found to be the same in tumor and normal tissues (Fig. 3), which could suggest such a signaling role, given the fact that phosphorylation of keratin proteins does occur [Baribault et al., 1989; Yano et al., 1991].

## ACKNOWLEDGMENTS

We thank Prof. J. Torhorst from the Institute of Pathology at the University of Basel, Prof. F. Harder from University hospital of Basel for providing the human tissue samples; Drs. F. Bachmann and R. Bänziger for antibody against p150, and Dr. J. Hershey for anti-eIF3 antibody. We are grateful to Drs. Nancy Hynes and Jörg Hagmann at FMI for their help in proofreading the manuscript and their valuable suggestions. L. Lin acknowledges the support of an American Cancer Society Postdoctoral Fellowship.

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