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Protein–protein interactions between keratin polypeptides expressed in the yeast two-hybrid system

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Abstract

Keratin filaments are obligatory heteropolymers of type I and type II keratin polypeptides. Specific type I/type II pairs are coexpressed in vivo. In contrast, all type I/type II pairs assemble into filaments in vitro, but the different pairs have different stabilities as demonstrated by treatment with increasing concentrations of urea. We have used the yeast two-hybrid system to analyse type I/type II interactions in a cellular context. We measured interactions between two different keratin pairs and we confirm the findings that K6+K17 form very stable heterodimers whereas K8+K18 interactions were weaker. The deletion of head domains did not reduce the strength of type I/type II interactions. Rather, the affinities were increased and the differences between the two pairs were retained in headless mutants. These findings argue against a major role of the head domains in directing heterodimer interactions and in defining heterodimer stabilities. © 1998 Elsevier Science B.V.

Keywords: Intermediate filament; Keratin; Protein–protein interaction; Yeast two-hybrid system

1. Introduction

Keratins represent the largest and most diverse group of intermediate filament (IF) proteins (for recent reviews, see [1,2]). Based on size, isoelectric point and sequence homologies, keratins are divided into type I (smaller acidic keratins) and type II (larger neutral to basic keratins) subfamilies. Keratin polypeptides share the structural organisation of all IF proteins consisting of a central α-helical rod domain flanked by non-α-helical head and tail domains [3–7]. The head domains have been subdivided into three regions. The E1 and V1 subdomains differ markedly in size and sequence whereas the H1 subdomain, situated adjacent to the rod, shows high sequence conservation amongst the type II but not the type I keratins [8]. Keratin IFs are obligatory heteropolymers consisting of equimolar amounts of type I and type II polypeptides [9]. The heteropolymeric nature of keratin IFs is implied in the formation of the coiled-coil dimer [10–12] and in vitro studies showed that intact rod domains are sufficient for dimerisation. In the next step of filament assembly, the heterodimers form antiparallel tetramers, which have been trapped and biochemically characterized in 4 M urea or 2 M guanidine-HCl buffers [3, 13–16]. In vitro studies of the K1/K10 IF assembly process suggested the existence of further oligomeric species,

Abbreviations: IF, intermediate filament; β-gal, β-galactosidase; BD, DNA binding domain of transcription factor GAL4; AD, transactivation domain of transcription factor GAL4

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e.g. hexamers [13]. Stable tetramers have been isolated in vitro from isolated rod domains. Thus, head and tail domains seem not to be essential in this early step of polymerisation [14,15]. In contrast, lateral and longitudinal association depend on the end domains. Although the tail domain is not essential for IF formation in vitro [16–21] it seems to play a role in the lateral packing of protofilaments [22].

The role of the head domains in filament formation is more complex. It is further complicated by the fact that in the heteropolymeric keratin system not all head domains seem to be equal in function. For example, headless K14 could be incorporated into pre-existing filaments [23] and assembled with intact K5 into IF in vitro [11,20]. Headless K5 assembled only very poorly into IF when combined with either headless or intact K14 whereas a K5 deletion mutant lacking 50% of its head domain readily assembled with its full-length partner K14 suggesting a role for the conserved H1 motif [20]. Coexpression of headless K8 with headless K18 or headless K19 led to the formation of disperse non-filamentous structures whereas the cotransfection of one headless plus one full-length clone resulted in fibrillar or granular structures [19]. A direct role of the head domains in IF assembly was suggested from extensive in vitro studies with head-truncated K8 and K18 polypeptides. No mutant was able to form regular IF structures in vitro [24]. The loss of the H1 domain of K8 had a particularly strong effect on IF formation and the analysis of the soluble complexes formed by headless K8 with full-length K18 in 2 M guanidine-HCl showed a reduced stability of the tetramers. The aminoterminal domains are also thought to interact with other proteins in the cell. For instance an 18-amino acid residue stretch including the sequence GSRS conserved in type II epidermal keratin heads is thought to interact with a series of five GSRS repeats in the tail domain of desmoplakin [25].

The diversity of keratin IF proteins and the specificity of pairwise coexpression raises the question which parts of the molecules are responsible for recognition of the correct partner keratins. Sorting between type III and type I polypeptides seems to depend on the rod domains as demonstrated by K14/vimentin hybrid polymerisation studies [26]. In this study, we have used the yeast two-hybrid system to compare interactions between type I/type II keratin pairs known to display different stabilities in urea buffers. We confirm that keratins K17/K6 interact very strongly whereas the K18/K8 interactions were weaker. Using deletion mutants we have analysed possible roles of the head domains in heterodimer formation and in determining stabilities of the coiled-coils. Differences in interaction strengths between K17/K6 and K18/K8 were retained when deletion mutants were coexpressed. Our data argue against an active role of the head domains in the early stages of keratin filament assembly.

2. Materials and methods

2.1. Yeast strain and culture media

The *Saccharomyces cerevisiae* strain YRG-2 (Matα *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3112 gal4-542 gal80-538* LYS::UAS_{GAL1}-$TATA_{GAL1}$-HIS3 URA3::$UAS_{GAL4\ 17mers(x3)}$-$TATA_{CYC1}$-*lacZ*) used in all experiments was purchased from Stratagene Cloning Systems (La Jolla, CA, USA). This strain is auxotroph for leucine, tryptophan and histidine. Yeast prepared for transformation were cultured at 30°C either on YPD agar plates or in YPD broth. Transformants were grown in synthetic dropout (SD) liquid medium and on SD agar plates supplemented with 10% (v/v) 10× dropout solution lacking either L-leucine and L-tryptophan (SDglc-LW) or L-leucine, L-tryptophan and L-histidine (SDglc-HLW). Transformants prepared for measurements of β-gal activity were shifted from SD liquid culture with 2% (w/v) glucose (SDglc-LW) to SD medium with 2% (w/v) galactose (SDgal-LW) to release any residual repression of GAL4 dependent reporter gene expression by glucose. For a detailed description of culture media see [27].

2.2. Yeast transformation and β-galactosidase (β-gal) assays

YRG-2 transformation was carried out using the Electroporator II apparatus (Invitrogen, De Schelp, The Netherlands). Electrocompetent cells were pre-

pared as suggested by the manufacturer. For cotransformation, 40 μl of electrocompetent cell suspension in 1 M sorbitol and 50 ng per plasmid DNA were combined in a sterile electroporation cuvette and incubated on ice for 5 min. Electroporation was carried out at 7.5 kV/cm with a loading capacitance of 50 μF and a loading resistance of 100 Ω. A 0.5 ml amount of 1 M sorbitol was added to the electroporated cells. Aliquots of 250 μl of this suspension were plated on SDglc-LW to screen for positive cotransformants and on SDglc-HLW to test qualitatively for interaction of the two-hybrid proteins encoded by the plasmids that were introduced. For quantitative measurements of β-gal activity the fluorometrical method of Meng et al. [28] was used with the following modifications: per cotransformant, a 1 ml SDglc-LW culture was inoculated with a single colony and grown to an OD at 600 nm of 1.5 (±0.05). The cells of 100 μl of this culture were harvested, resuspended in the same volume of SDgal-LW and incubated for 1 h at 30°C with shaking. The cells were harvested again and utilized further as described in [28]. Three independent cotransformants were used and fluorescence was measured with a LS-50B luminescence spectrometer (Perkin–Elmer, Norwalk, CT, USA).

2.3. Indirect immunofluorescence

A 5 ml culture of a cotransformant in the appropriate medium was grown at 30°C to an OD at 600 nm of 0.3–0.4. Formaldehyde was added to a final concentration of 3.7% and cells were fixed for 1 h at 30°C. Cells were pelleted and washed once with 5 ml 100 mM potassium phosphate, pH 7.4. Spheroblasts were prepared by resuspending the cells in 1 ml of the same buffer containing 0.2% mercaptoethanol and 50 U/ml lyticase and incubation for 30–45 min at 30°C. Degradation of cell walls was confirmed by microscopic inspection of an aliquot stained with Methyl blue. Spheroblasts were collected and resuspended in 1 ml PBS. A 15 μl amount of each suspension was added to a well of a poly-L-lysine-coated 10-well slide, and cells were allowed to attach for 15 min. The supernatant was removed and PBS/1% BSA was added for 30 min to block unspecific binding sites. The blocking solution was removed and the primary antibody added. Incubation was at room temperature for 45 min in a dark moist chamber. After removal of the antibody each well was washed 10 times with PBS/BSA, and the secondary antibody was applied. The wells were washed again with PBS/BSA and twice more with PBS. For localisation of nuclei, cells were stained for 5 min at room temperature in the dark with DAPI (1 μg/ml in PBS). After washing twice with PBS slides were mounted in Mowiol.

Primary antibodies were: polyclonal antibodies GAL4-DBD and GAL4-TA(768) directed against the DNA binding domain and transactivation domain of GAL4, respectively (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Both antibodies were diluted to 2 μg/ml. The secondary antibody Cy3-coupled anti-rabbit IgG was used at a dilution of 1:1000 and purchased from Dianova, Hamburg, Germany.

2.4. Yeast vectors and plasmid construction

Phagemid shuttle vectors containing the GAL4 DNA-binding domain (BD) pBD-GAL4 (pBD) and the GAL4 transactivation domain (AD) pAD-GAL4 (pAD) as well as control plasmids p53 and pSV40 were purchased from Stratagene Cloning Systems (La Jolla, CA, USA). Cotransformation of control plasmids p53 (expressing the hybrid of BD of GAL4 and amino acids 72–390 of murine p53) and pSV40 (expressing the hybrid of AD of GAL4 and amino acids 84–708 of SV40 large T-antigen) served as a positive control. For a detailed description of the vectors see [27]. Plasmids for high level expression of the same p53-BD and SV40-AD constructs in yeast (pVA3-1 and pTD1-1, respectively) were from Clontech (Palo Alto, CA, USA). The cDNAs encoding the full length and several deletion mutants of K17, K18, K6 and K8 (for a schematic representation see Fig. 1) were obtained by RT-PCR on total RNA from human A431 (K17, K6) and MCF-7 cells (K18, K8), respectively. Total RNA was prepared according to the protocol of Auffrey and Rougeon [29]. First strand cDNA synthesis was performed with 5 μg total RNA using the 1st Strand cDNA Synthesis Kit from Pharmacia Biotech (Uppsala, Sweden) following the manufacturer's instructions. Sense primers were designed with *Eco*RI recognition sites and antisense primers introduced a *Sal*I site into the PCR fragments. The amplification products were

purified from preparative agarose gels and ligated into the pCR 2.1 vector (Invitrogen, De Schelp, the Netherlands). The identity of the cDNA clones was verified by automated sequencing (model 373a, Applied Biosystems, Foster City, CA, USA). DNA from positive clones was digested with *Eco*RI and *Sal*I, respectively, and the resulting inserts were cloned into the unique *Eco*RI and *Sal*I sites of pBD and pAD.

Table 1
Control measurements for β-gal activities

Insert in pAD	Insert in pBD	Fluorescence (mean ± S.D.; arbitrary units)
None	None	10.6 ± 2.1
SV 40 large T	p53	66.9 ± 7.2
K17	None	20.9 ± 3.2
None	K17	20.5 ± 3.3
K17-H	None	16.2 ± 2.9
None	K17-H	14.7 ± 1.3
K17-HL	None	19.5 ± 2.5
None	K17-HL	18.2 ± 2.0
K18	None	4.8 ± 0.4
None	K18	3.4 ± 0.8
K18-H	None	4.4 ± 1.6
None	K18-H	5.3 ± 2.8
K18-HL	None	1.7 ± 0.8
None	K18-HL	2.8 ± 1.5
K6	None	2.1 ± 0.3
None	K6	3.8 ± 0.6
K6-H	None	11.3 ± 2.3
None	K6-H	10.9 ± 2.2
K6-H1	None	4.7 ± 1.8
None	K6-H1	7.0 ± 0.8
K6-HL	None	16.2 ± 1.7
None	K6-HL	13.5 ± 0.7
K8	None	5.2 ± 1.3
None	K8	4.6 ± 0.8
K8-H	None	3.7 ± 0.8
None	K8-H	4.2 ± 0.3
K8-H1	None	3.6 ± 1.5
None	K8-H1	4.2 ± 1.8
K8-HL	None	2.6 ± 0.6
None	K8-HL	1.6 ± 0.7

The relative fluorescence values shown are the means ± S.D. of at least three independent experiments. SV40 large T-antigen and p53 served as a positive control for the system.

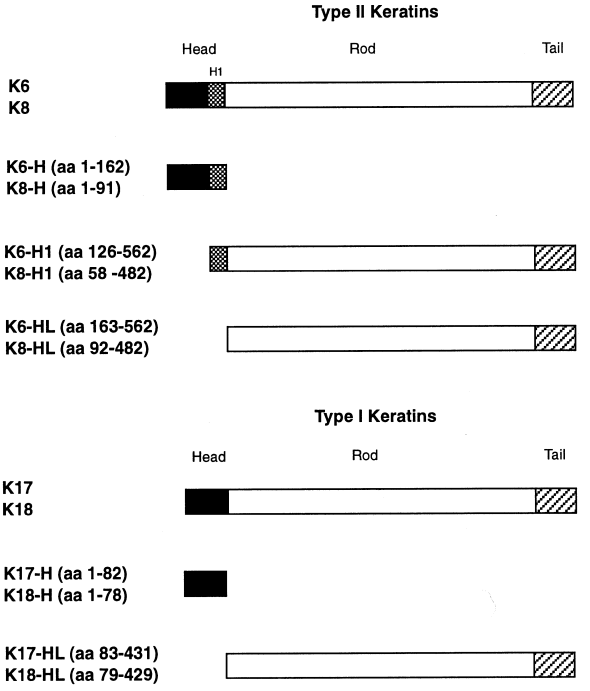

Fig. 1. Schematic representation of the cDNA clones used in this study. Clones comprise full-length keratins, headless (HL), partially headless type II deletion mutants (starting with the H1 domain, H1) and head domains (H). Numbers in brackets refer to the amino acid residues (aa) of the full-length polypeptide.

3. Results

In order to compare the strength of interactions of different keratin pairs we chose the two keratin pairs K17/K6 and K18/K8. The K17/K6 pair is expressed mainly in specific skin appendages, developing interfollical epidermis and cultured keratinocytes [30–32]. This pair is highly resistant to denaturation and builds complexes even in 9 M urea buffer (our unpublished observations). In contrast, the K18/K8 pair is expressed in simple epithelia [30] and K18/K8 complexes can be dissociated into monomers by 7.5 M urea [9].

The influence of the head domains on the relative binding strength between individual keratins was examined by expressing both full-length and truncated polypeptides from the K17/K6 and K18/K8 pairs in the yeast two-hybrid system. Using a fluorescence based β-gal assay, we quantified the β-gal activity which is used as a reporter gene for interacting hybrid proteins. The interaction between p53 and SV40 large T-antigen was analysed for comparison. As

shown in Table 1, cotransformation of the control plasmids led to a 6.5-fold increase in fluorescence compared to results obtained when cotransformation of the corresponding vectors without the insert was performed. Coexpression of the full-length keratin pair K17/K6 or of the full-length K18/K8 keratin pair resulted in similarly high β-gal activities (Fig. 2a). We conclude that interactions between pairs of keratin polypeptides can be detected in the yeast two-hybrid system and that the binding strengths between full-length partner keratins are in the same range as those seen between p53 and SV40 large T-antigen. Reporter gene activation was similar when

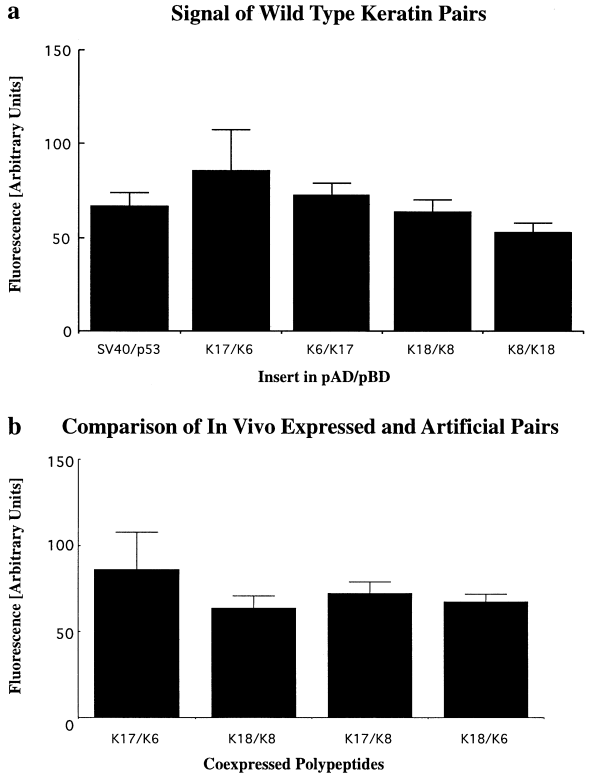

Fig. 2. Analysis of type I/type II keratin interactions. β-gal activities were measured after cotransformation of plasmids for expression of full-length keratin polypeptides. Data are the means of at least three independent measurements. Standard deviations are indicated. (a) Interactions between K17/K6 and K8/K18 which occur as partner keratins in vivo. p53/SV40 large T-antigen interaction is given for comparison. Swapping of the insert between the two vectors had only a minor influence on reporter gene activation. (b) Comparison of reporter gene activation due to interactions between artificial and natural keratin pairs. In this experiment, the type I keratins were expressed as the AD hybrid proteins whereas the type II polypeptides were expressed as the BD hybrids.

keratin constructs were fused to either GAL4 domain. In the following experiments, we show β-gal activities of cotransformants expressing the type I polypeptides as the AD hybrid proteins and the type II polypeptides as the BD hybrid proteins.

Strong interactions were not restricted to the natural partners K17/K6 and K18/K8. The artificial pairs K17/K8 and K18/K6 showed almost as high affinities as the natural pairs (Fig. 2b). Interaction strengths were intermediate between the natural pairs but seemed to be dictated by the type I keratin as β-gal activities were higher when K17 was the partner.

The influence of the type I head domains on binding to full-length type II keratins is summarized in Fig. 3a. Coexpression of type I head domains with its full-length partner did not lead to an increased β-gal activity and cotransformants did not grow on plates lacking histidine (data not shown). In contrast, truncation of the type I head domains seemed to strengthen the interaction with the partner keratin. This effect was very pronounced in the highly stable K17/K6 pair. In the case of the K18/K8 pair, a slight increase in fluorescence intensity was observed.

Like the type I head domains, the type II head domains did not lead to a substantial β-gal activation when coexpressed with the full-length partner keratins (Fig. 3b). The deletion of the entire type II head led to an increase in binding to the full-length type I keratin as indicated by a 1.5-fold increase of reporter gene activity for K17/K6 and a 2-fold increase for K18/K8. The highest β-gal activities were measured when full-length type I keratins were combined with the partner type II polypeptides lacking the variable regions of the head domains, but retaining the highly conserved H1 domains. This suggests that the H1 domain might play a role in dimer and/or tetramer formation. This effect was again more pronounced for the K17/K6 pair as compared to the K18/K8 pair.

The combination of two headless keratins also led to an increased binding strength when compared to the full-length polypeptides (Fig. 3c). Again interactions between K17/K6 headless keratins were stronger than those between K18/K8 headless keratins. However, interactions between two headless keratins were weaker than those between one headless and one full-length keratin.

Somewhat surprising results were observed when

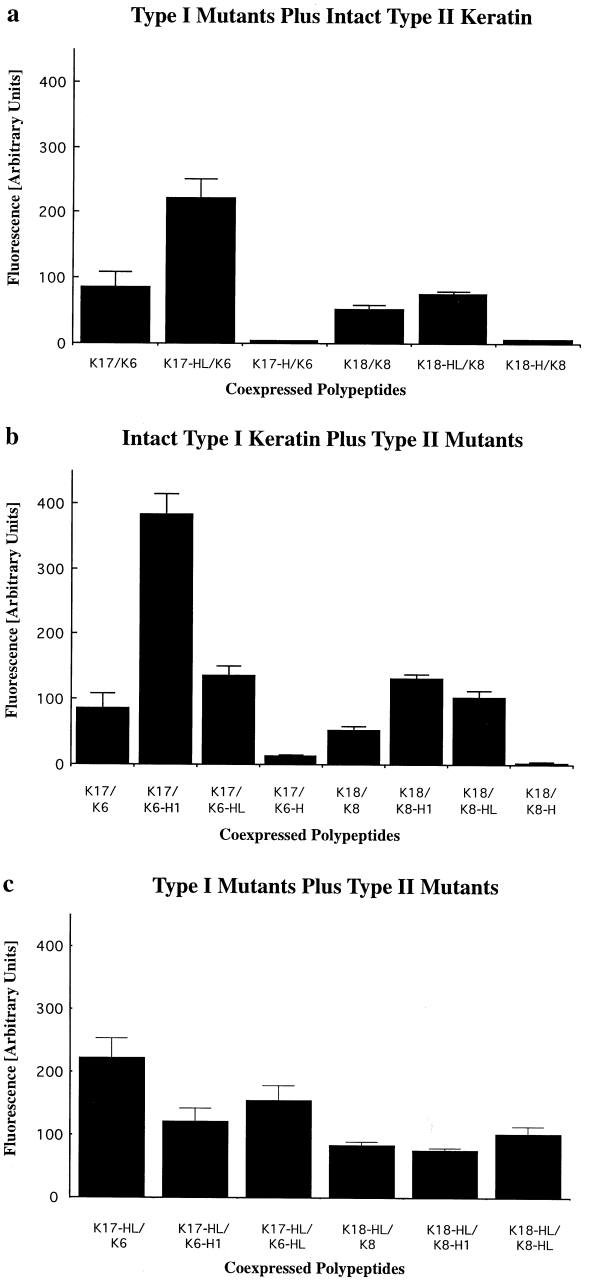

Fig. 3. Interactions of headless keratins and head domains. (a) Interactions between type I mutants and intact type II keratins. Headless type I polypeptides (HL) and head domains (H) were expressed as the AD hybrid proteins, whereas type II keratins were expressed as the BD hybrid proteins. (b) Interactions between headless type II keratins (HL), partially head deleted mutants (H1) and type II head domains (H) with their full-length type I partner keratin. (c) Interactions between headless type I and headless type II keratins.

homotypic interactions of the keratins were examined (see Fig. 4). Full-length type I keratins as well as headless type I polypeptides showed homotypic interactions and the reporter gene activation was in the same range as observed for type I/type II interactions. Truncation of the head domains did not lead to an increase in β-gal activities as observed for heterotypic interactions, but instead to a slight decrease. In contrast to type I keratins, homotypic interactions between the type II keratins were not detected. Neither K6 nor K8 nor mutants of these polypeptides showed self association.

The observed differences in β-gal activities could be due either to different affinities of the constructs or they could be caused by substantially different expression levels and diminished β-gal activation as a consequence of filament formation or keratin aggregation in the cytoplasm of the yeast cells. To investigate this we performed indirect immunofluorescence staining of cotransformants with antibodies against the GAL4 domains (Fig. 5). Cotransformants

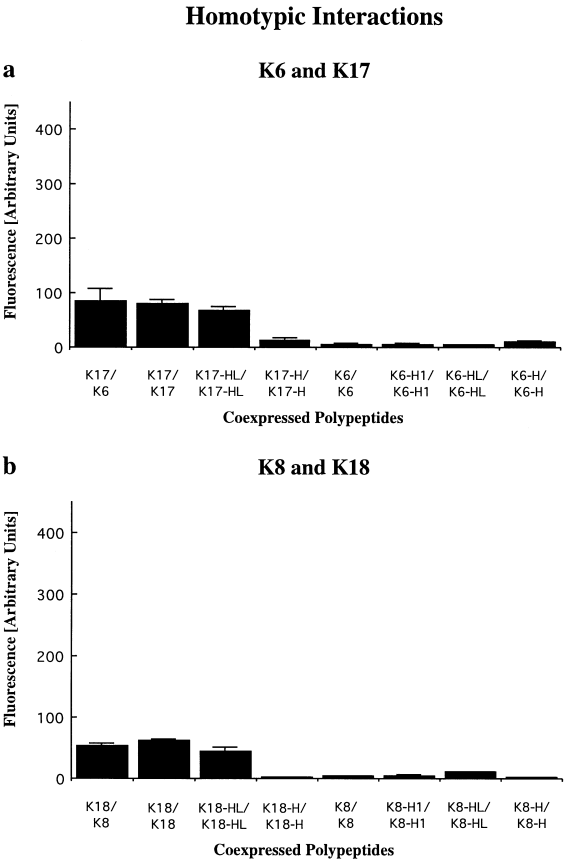

Fig. 4. Homotypic interactions of type I and type II keratins and their deletion mutants. (a) K17 and K6 interaction. (b) K18 and K8 interactions.

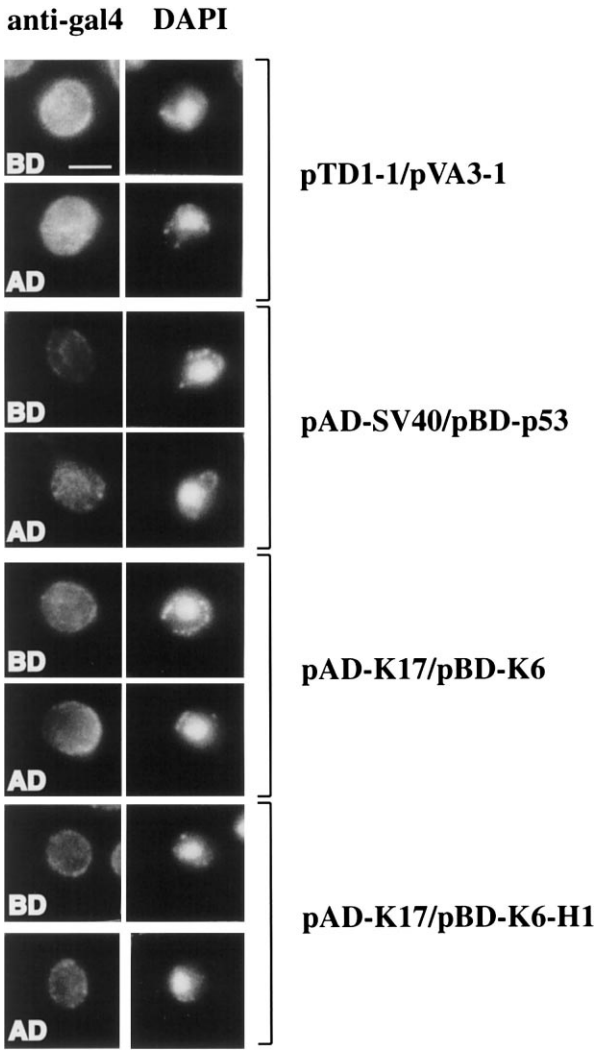

Fig. 5. Immunofluorescence staining of yeast cells cotransformed with two-hybrid constructs. Cells were cotransformed with the plasmids indicated at the right. After fixation with formaldehyde cells were stained with antibodies against the binding domain (BD) of GAL4, the transactivation domain (AD) of GAL4 as indicated (left panel) and double stained for localization of nuclei with DAPI (right panel). Scale bar: 1 μm.

expressing SV40 large T-antigen/p53 showed a strong staining when vectors allowing high expression were used (pTD1-1/pVA3-1). In contrast, expression of the same constructs in the low expression vectors used in our study led to substantially weaker staining (pAD-SV40/pBD-p53). Aggregates or filamentous structures were not detected either in cotransformants expressing intact partner keratins or in cells expressing headless polypeptides. To control expression levels, we also performed Western blotting of cell extracts of the cotransformants with GAL4 antibodies. While expression of hybrid proteins was readily detectable when high expression vectors were used, expression of our constructs could not be detected (data not shown).

4. Discussion

In vitro studies on the early stages of keratin filament assembly starting with denaturating conditions always raise the question as to the relevance of these findings in an in vivo context. Therefore, we have used the yeast two-hybrid system to study early stages of keratin filament assembly in vivo [33].

The quantitative measurement of β-gal as a reporter gene in a fluorescence based assay allows a comparison of the relative binding strengths of the different partners [28]. However, the interpretation of β-gal activities only in terms of relative affinities ignores the fact that reporter gene activity may be influenced additionally by such factors as expression rates, nuclear import or improper folding of the coexpressed proteins. The GAL4 domains may interfere with binding to a coexpressed partner especially when assaying small fragments such as the keratin head domains used in this study. Thus, the lack of β-gal activity when these domains were expressed could be due to improper folding or to steric hindrance by the GAL4 domains which are approximately the same size. However, in spite of these restrictions reporter gene activities give a useful estimation of binding affinities in most cases [28,34–36].

Specific keratin pairs are characteristically coexpressed in different organs and tissues. In addition, each keratin pair displays typical properties as the solubility of filaments [37] and stability of heterodimers to denaturation with urea [9]. Whereas K17/K6 interact even in 9 M urea, K18/K8 dimers dissociate at lower concentrations of denaturating agent. We show that these different stabilities can be detected in an in vivo context. In addition, the diverse characteristics of keratin pairs cannot be attributed to the aminoterminal domains since the deletion of head domains did not abolish or reduce binding of the rod domains and differences in affinities between the K17/K6 pair and the K18/K8 pair were retained.

Instead, deletion of the aminoterminal domains of a partner keratin pair increased reporter gene activity when compared to the full-length keratin pair (Fig. 3). This result could be due to low expression, nuclear transport rates and/or intermediate filament formation or aggregation when full-length proteins were coexpressed. Indirect immunofluorescence staining of yeast cells cotransformed with vectors for expression of the full-length pair gave no indication for the formation of filaments or of aggregates (Fig. 5). Moreover, control proteins (SV40 large T-antigen+p53) as well as keratin polypeptides were not detectable in yeast cell extracts by Western blotting confirming that expression levels were low and suggesting that keratin levels did not reach the critical concentration for filament formation [13]. Therefore, we conclude that the differences in β-gal activities we detected represent primarily differences in binding strengths between the two-hybrid constructs and not differences in expression, impaired nuclear import due to filament formation or steric hindrance.

In all cases examined, deletion of the head domains led to an increase in reporter gene activation compared to full-length keratins (Fig. 3). At the level of dimer formation the destabilizing effect of head domains on type I/type II interactions can be explained either by repulsion between the positively charged head domains or by intramolecular interactions between head and rod domains. These interactions would block binding sites for the partner keratin and consequently reduce heterotypic interactions. The hypothesis that intramolecular interactions between rod and head domains may weaken heterotypic interactions is supported by the finding that head domains are extremely prone to degradation when artificially fused to the rod domain of the partner keratins suggesting that the head domain is normally stabilized through an interaction with the rod domain [24]. The strongest increase in reporter gene activity was observed for type II keratins which lacked only the variable portion of the head domains, but retained the conserved H1 sequence motif (see Fig. 3b). Additional deletion of the H1 domain reduced the binding to the full-length partner keratin (Fig. 3b). These binding characteristics suggest that the H1 domain plays an active role in dimer formation in agreement with in vitro assembly studies of head truncated mutants [24]. The H1 domain probably binds to the type I head domain thereby releasing intramolecular head–rod interactions in the partner and at the same time aligning the two polypeptide chains to facilitate subsequent coiled-coil formation. This interpretation is in agreement with the finding that further deletion of the H1 domain reduces binding to the full-length partner. Type II keratins lacking the H1 domain bind with similar strength to full-length and headless partner keratins (compare Fig. 3b and c). On the other hand, type II mutants which include the H1 domain show a much stronger interaction with the full-length type I keratin than with the headless mutant (compare Fig. 3b and c) which lacks the putative binding site for the H1 domain.

Taken together binding characteristics of headless mutants can be explained by two effects: (1) intramolecular interactions between head and rod domains mask binding sites involved in coiled-coil formation and consequently reduce interactions between full-length keratins compared to headless keratins; and (2) the H1 domain of type II keratins binds to the type I head. As a consequence intramolecular interactions of head and rod domains are released in the partner and, furthermore, both polypeptide chains are aligned in parallel and in register thus facilitating coiled-coil formation.

Meng et al. [28] showed by crosslinking that vimentin constructs expressed in the two-hybrid system formed tetramers. Since keratins behave similarly, our observations may reflect interactions between individual polypeptides and interactions between dimers. If tetramer formation stabilizes hybrid complexes and increases reporter gene activity, head domains would decrease β-gal activity by weakening the interaction between coiled-coil dimers. In contrast to this hypothesis, in vitro analysis of K18/K8 in buffers containing 2–4 M guanidinium hydrochloride revealed a stabilizing effect of head domains [24]. Moreover, studies using synthetic peptides showed that the H1 domain is essential for alignment of coiled coils and has a stabilizing effect. [38]. Therefore, we conclude that the different binding characteristics of headless and full-length keratins predominantly reflect effects of mutations on coiled-coil formation and only to a minor degree on tetramer formation.

In addition to affinities of the keratin pairs ex-

pressed in vivo, we also examined interactions of the artificial pairs K17/K8 and K18/K6. These interactions were intermediate between the strong K17/K6 pair and the weaker K18/K8 pair (see Fig. 2b). Similar results were found in vitro when stabilities of the pairs K14/K5 and K10/K1 were compared with those of K14/K1 and K10/K5 [9].

In our study, type I keratins interacted almost as strongly with type I keratins as they did with the type II partners. In contrast, type II keratins as well as truncated type II polypeptides showed no homotypic interactions (Fig. 4). In a similar study by Meng et al. [28], heterotypic interactions between K18 and K8 were much stronger than homotypic interactions. The reasons for this discrepancy are presently unknown. Previous studies support our findings that type I keratins form homodimers and behave differently from type II polypeptides: whereas type I keratins assembled into small soluble oligomeric rod-like structures in vitro, type II molecules formed bold-like structures and insoluble precipitates [39]. Moreover, our results on the lack of homotypic interactions of type II keratins are consistent with in vitro binding studies under non-denaturating conditions using plasmon resonance measurements in which no K8/K8 interactions were detected [40]. On the other hand, analytical ultracentrifugation and CD spectroscopy revealed that both type I and type II keratins form homodimers in buffer containing 3 M guanidinium hydrochloride [41]. Also homodimer formation was observed in crosslinking experiments with cysteine containing K18 and K8 mutants in vitro [10]. However, cystine formation does not depend on coiled-coil formation and since dimers cannot dissociate in this case, the equilibrium is shifted towards the dimer, even if the interaction is weak and/or transient.

A stabilisation of type I keratins as homodimers seems to be reasonable in an in vivo setting where type II keratins are expressed in excess over type I keratins [42–46]. Type I expression seems to be tightly regulated and these keratins are transiently stabilized as homodimers whereas the excess of type II keratins is rapidly degraded. Thus, type I homodimers may serve as matrices for assembly with the type II keratins.

Yeast two-hybrid experiments examining the role of end domains in filament assembly were also conducted with lamins [47] and neurofilament proteins [35,36]. These studies showed that neurofilament head domains were responsible for the specificity of the heterodimer interaction. Our data showing that keratin binding specificities are not dictated by their head domains confirm the fundamental differences between the keratin filaments and the neurofilaments. Whereas keratins are obligatory heteropolymers built from type I/type II heterodimers [10,48] neurofilaments are assembled from NF-L and variable amounts of NF-M and NF-H [49].

Taken together, we have shown that keratin interactions can be analysed in an in vivo context using the yeast two-hybrid system. We confirm the different stabilities of specific keratin pairs found in vitro and demonstrate that binding characteristics are retained in headless mutants. Therefore, we conclude that the head domains do not play a critical role in directing heterotypic subunit formation. Moreover, we show that type I keratins form homodimers in vivo and confirm findings that type I and type II keratins behave differently.

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