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Novel subunits of the TATA binding protein free TAF_{II}-containing transcription complex identified by matrix-assisted laser desorption/ionization-time of flight mass spectrometry following one-dimensional gel electrophoresis

Initiation of transcription of protein-encoding genes by RNA polymerase II was thought to require the transcription factor II D ($TF_{II}D$), a complex comprising the TATA binding protein (TBP) and TBP-associated factors. However, another multiprotein complex isolated more recently and called TFTC (TBP-free TAF_{II} containing complex), was shown to mediate initiation of RNA polymerase II (Pol II) transcription in the absence of $TF_{II}D$ as well as specific acetylation of histone H3 in a nucleosomal context. Several subunits of the TFTC complex were already identified using classical methods such as Edman based microsequencing and Western blot analysis. In this article we present a mass spectrometry based proteomic approach to confirm previous results and to identify other possible subunits of the TFTC complex. The TFTC complex was separated on one-dimensional sodium dodecyl sulfate polyacrylamide electrophoresis and analysed by matrix-assisted laser desorption/ionization-time of flight mass spectrometry and peptide mass fingerprinting. Identifications were realized after databank searches. This new characterization of TFTC complex confirmed the presence of already described subunits (TRRAP, GCN5, SAP130/KIA0017, $TAF_{II}150$, $TAF_{II}135$, $TAF_{II}100$, $TAF_{II}80$, $TAF_{II}20$, SPT3 and PAF65β). Moreover, a good coverage of these sequences was obtained. Interestingly, $TAF_{II}32$ and PAF6α were also determined as potential novel subunits of TFTC. These results together show the suitability and the great potential of this method and offer new perspectives in fundamental studies of transcription factor complexes.

Keywords: Matrix-assisted laser desorption/ionization-time of flight mass spectrometry / One-dimensional gel electrophoresis / Transcription factors / TATA binding protein-free TAF containing complex / TATA binding protein associated factor PRO 0344

1 Introduction

Transcription initiation of protein-encoding genes by RNA polymerase II (Pol II) was thought to require the transcription factor II D ($TF_{II}D$), a protein complex comprised of the TATA binding protein and series of TATA binding protein (TBP)-associated factors ($TAF_{II}s$) [1, 2]. However, we have recently shown that initiation of Pol II transcription can occur in the absence of $TF_{II}D$, in the presence of a novel human (h) multiprotein complex, termed TFTC (TBP-free TAF_{II} containing complex) [3]. TFTC is able to direct preinitiation complex assembly on both TATA-containing and TATA-less promoters *in vitro*. TFTC contains neither TBP nor TBP-like factor, but is composed of several $TAF_{II}s$ [3]. The three-dimensional structure of TFTC has been determined, together with that of $TF_{II}D$, at 3.5 nm resolution by electron microscopy and digital image analysis of single particles [4]. Human TFTC resembles a macromolecular clamp that contains five globular domains organized around a solvent accessible groove of a size suitable to bind DNA. $TF_{II}D$ contains only four domains, which are also organized around a solvent accessible groove [4, 5]. Comparison of the two 3-D models indicates that the structure of $TF_{II}D$ is almost included in that of TFTC, further confirming some of the described functional similarities.

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Abbreviations: TAF, TBP associated factor; **TBP**, TATA binding protein; **$TF_{II}D$**, transcription factor II D; **TFTC**, TBP-free TAF containing complex

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TFTC, similar to other TBP-free TAF_{II} complexes, including yeast SAGA, hSTAGA and hPCAF/GCN5, contains the acetyltransferase hGCN5 and is able to acetylate histone H3 in both a free and a nucleosomal context [6–10]. The fact that histone acetylation has been linked to the activation of transcription [11] suggests that TFTC is recruited to chromatin templates by activators to acetylate histones and potentiate the initiation of transcription [3, 6]. Additional TFTC subunits common to other human TAF_{II}-HAT (histone acetyltransferase) complexes have been identified, including hADA3, $hTAF_{II}150$, hSPT3, hPAF65β, TRRAP and SAP130/KIA0017 [6, 12].

Transcription factors are very difficult to identify because of their very low abundance in a cell. It became important to find new analytical tools in term of sensitivity. Technological advances during recent years, have reoriented the strategy of protein identification. First, with the help of large-scale genomic and cDNA sequencing projects many novel protein sequences have been determined from different species and stored in large databases. Moreover, there was a very rapid development of new mass spectrometric techniques which allow accurate mass measurements of peptides and proteins at low concentration.

Mass spectrometry has become an important tool in this post-genomic era, first by the development a few years ago of MALDI-TOF MS, which is to date widely used as a direct tool in the identification of protein separated by 2-DE [13–15]. MALDI-TOF MS data combined with on line databank searches have revolutionized rapid protein identification. We have taken the advantage of the efficiency of this approach to identify subunits of low abundance transcription factor complexes. Even though some of the subunits of TFTC were already identified [3, 6], we wanted to confirm these results by a second method using a proteomic approach and to identify novel subunits of TFTC.

Proteins were separated by a 1-D SDS-PAGE and visualized by Coomassie blue staining. Colored polypeptides were cut out from the gels and treated (see Section 2.3). After in-gel tryptic digestion, extracted peptides were deposited onto a MALDI target and their mass determined by MALDI-TOF MS measurements, using the highest possible accuracy available with our instrument (± 20 ppm). The results obtained not only confirmed the presence of several already described subunits of TFTC but also allowed us to identify two novel subunits of the complex. Moreover, we obtained significant protein coverages indicating a high reliability of the protein identification performed according to the approach based MS. Thus, this study highlights the advantages of the use of MALDI-TOF MS in terms of sensitivity and rapidity, when compared to more traditional methods. This study was performed on the same samples with both the MS approach and Western blot analysis. The complete agreement between the results obtained with the two methods makes our identifications particularly reliable.

2 Materials and methods

2.1 Immunoprecipitation

MAbs used for immunoprecipitation (IP) were purified from ascites fluids using caprylic acid precipitation followed by precipitation with 50% ammonium sulphate and dialysis. TFTC preparation was described in Wieczorek *et al.*, [3]. Briefly, 100 mg (from 5×10^9 cells) of HeLa nuclear extract [16] was first passed through a single ssDNA cellulose column to eliminate proteins which bind nonspecifically to the resin used later in immunoprecipitations. Fractions were immunoprecipitated with protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden) bound mAbs, first with an anti-$TAF_{II}30$ mAb and second with an anti-TBP mAb. The protein G-Sepharose antibody bound complexes were washed $3\times$ with IP buffer (25 mM Tris-HCl pH 7.9, 10% v/v glycerol, 0.1% NP-40, 0.5 mM DTT, 5 mM $MgCl_2$ containing 0.5 KCl and $2\times$ with IP buffer containing 100 mM KCl. After washing, the bound proteins were eluted with a 200–1000 fold excess of the corresponding epitope peptide, centrifuged using a Nanosep 30 kDa cut-off centrifugal concentrator (Pall Gelware, St. Germain en Laye, France), and analyzed by SDS-PAGE. The gels were either stained with Coomassie colloidal Blue (Bio-Rad, Hercules, CA, USA) or transferred to nitrocellulose membrane and probed with the relevant antibodies.

All mass spectral data was obtained from the same preparation of the TFTC complex, corresponding to approximately 27 μg of the 2 MDa complex (estimated by comparison with BSA protein stained with Coommassie colloidal Blue after separation by SDS-PAGE).

2.2 Western blot analysis

Western blots were carried out by standard procedures and treated with either polyclonal rabbit or monoclonal mouse antibodies followed by incubation with peroxidase-conjugated goat-antirabbit or goat-antimouse secondary antibodies (Jackson Immuno Research, West Grove, PA, USA). Chemiluminescence detection was performed according to the manufacturer's instructions (Amersham Biosciences). Polyclonal antibodies against hGCN5, TRRAP, and the mAbs against $TAF_{II}20$, $TAF_{II}30$, $TAF_{II}100$ and $TAF_{II}135$ have been previously characterized

and described [12]. The polyclonal antibody raised against hTAF$_{II}$150 was a kind gift from S. Smale. The polyclonal antibodies against hADA2, hADA3, hPAF65β were raised against bacterially produced recombinant proteins (a kind gift from Y. Nakatani).

2.3 In-gel digestion procedure

Each gel slice was cut into small pieces with a scalpel, washed with 100 μL of 25 mM NH_4HCO_3, and agitated for 8 min with a vortex mixer. After removal of the supernatant, gel pieces were dehydrated with 100 μL of acetonitrile, agitated for 8 min with a vortex mixer. The supernatant was again removed. This operation was repeated three times [17]. Gel pieces were completely dried with a SpeedVac (15 min) and were covered with 100 μL of 10 mM DTT in 25 mM NH_4HCO_3 for the reduction step, the reaction was left to proceed at 57°C for 1 h. After the removal of the supernatant, 100 μL of 55 mM iodoacetamide in 25 mM NH_4HCO_3 were added and the reaction was left to proceed in the dark at room temperature for 1 h. The supernatant was removed and the washing procedure with 100 μL of NH_4HCO_3 and acetonitrile was repeated three times. Gel pieces were completely dried with a SpeedVac before tryptic digestion and swelled in a solution of trypsin (12.5 ng/μL, Promega, Madison, WI, USA) in 25 mM NH_4HCO_3. The digestion was performed at 35°C overnight. The gel pieces were centrifuged, 5 μL of 25% H_2O/70% acetonitrile/5% HCOOH were added. The mixture was sonicated for 5 min and centrifuged. The supernatant was recovered and the operation was repeated once. The supernatant volume was reduced under nitrogen flow to 4 μL after adding 1 μL of H_2O/5% HCOOH, 0.5 μL were used for MALDI-TOF analysis.

2.4 MALDI-TOF MS

Mass measurements were carried out on a Bruker BIFLEX III MALDI-TOF Mass Spectrometer equipped with the SCOUT (Bruker, Bremen, Germany), high resolution optics with X–Y multisample probe and griddles reflector. This instrument was used at a maximum accelerating potential of 19 kV and was operated in reflector mode. Ionization was accomplished with a 337 nm beam from a nitrogen laser with a repetition rate of 3 Hz. The output signal from the detector was digitized at a sampling rate of 2 GHz. A saturated solution of α-cyano-4-hydroxycinnamic acid in acetone was used as a matrix. A first layer of fine matrix crystals was obtained by spreading and fast evaporation of 0.5 μL of matrix solution. On this fine layer of crystals, a 0.5 μL droplet of aqueous HCOOH (5%) solution was deposited. Afterwards, 0.5 μL of sample solution was added and a second droplet 0.2 μL of matrix saturated solution (in 50% H_2O/50% ACN) was added. The preparation was dried under vacuum. Before the deposit the samples were concentrated between five- and ten-fold on C18 Zip-Tips (Millipore, Bedford, MA, USA). The sample was washed one to three times by applying 1 μL of aqueous HCOOH (5%) solution on the target and then flushed after a few seconds. The calibration was performed in internal mode with four peptides, angiotensin at *m/z* 1046.542, Substance P at *m/z* 1347.736, Bombesin at *m/z* 162 0 807 and trypsin autolysis fragment at *m/z* 2211.107.

2.5 Database searches

Ions obtained from MALDI spectra were directly used for database searches using the software MS-Fit developed at the UCSF mass spectrometry facility and available on the internet to search against proteomic databases (SWISS-PROTand NCBInr). Database searches were performed using the following values, protein molecular mass of 10–200 kDa, trypsin digest with one missed cleavage allowed, cysteines modified by carbamidomethylation, mass tolerance of 50 ppm using internal calibration and oxidation of methionines. The identification was based upon at least four matching peptides.

3 Results and discussion

3.1 Identification of TFTC subunit composition using MALDI-TOF MS analysis

The TFTC complex was purified as described previously [3]. The TFTC complex (27 μg) was separated by 1-D SDS-PAGE, because of the limited number of components, and stained with colloidal blue. About 14 bands ranging from approximately 20–300 kDa were visualized (Fig. 1). Two experiments were performed in parallel in order to compare the reproducibility of the gels. The gel pieces containing the colored polypeptides were excised and washed. Following reduction alkylation and several washing steps, which were crucial before tryptic digestion to obtain keratin free samples, the resulting peptides were extracted (see Section 2.3). Concentration and desalting steps using ZipTips have considerably improved the signal-to-noise ratio and enabled the measurement of a wide peak distribution in a narrow mass range in MALDI measurements [18]. Although suppression effects are often described in the MALDI process [19], we were able to observe a high number of molecular ions, as shown in Fig. 2, where as many as 107 peaks were obtained from m/z 750–2400. These data permitted the identification of a 190 kDa protein called clathrin (a con-

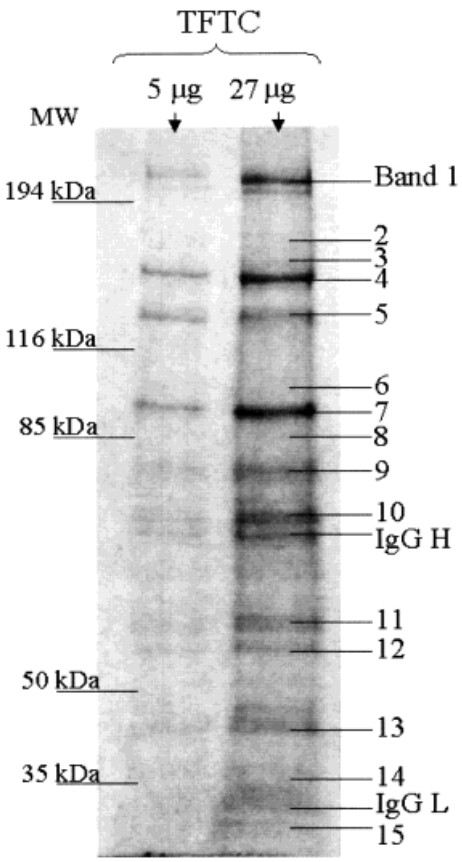

Figure 1. Separation of the subunits of the TFTC complex by SDS-PAGE. The subunits of the TFTC complex (5 µg and 27 µg) were separated by an 8% SDS-PAGE, visualized by Coomassie brillant blue staining, the bands cut and processed for MALDI-TOF MS analysis. The positions of the heavy (IgG H) and the light (IgG L) chain of the antibody are indicated.

taminating protein species in the TFTC preparation that copurifies with the mouse mAb used for the immunopurification of TFTC (data not shown)). Moreover, we obtained 28% of sequence coverage as well as a mass accuracy average of 21 ppm, which are very good values for a protein of 190 kDa. Thus, the MS approach has permitted the identification of an unexpected protein species which has not been detected with the Western blot approach used previously.

These results show that even in the case of a large number of peaks in a MALDI spectrum, the mass measurements were of good quality. After careful analysis of the obtained masses, it appeared that a number of peptides could not be assigned to clathrin. This can be explained by the fact that either (i) the database search identified the correct protein but some of the peptides were post-translationally modified, or (ii) the trypsin could have generated unspecific peptides, or finally (iii) because of the low resolution of a 1-D gel, several proteins could have comigrated in the same band [20]. For this reason we performed new database searches after subtracting the matched peptides of clathrin. With this approach we identified one additional protein in band 1 and two further proteins of interest in band 2. In bands 1 and 2, a total of 9 and 11 peptides respectively matched for TRRAP (a c-myc-interacting transcription coactivator), with a mass accuracy average of 21 ppm. Moreover, in band 2, seven peptides matched to $TAF_{II}150$ (a 150 kDa subunit of $TF_{II}D$) with a mass accuracy average of 12 ppm (Fig. 2). Even if the percentage of coverage was not really representative of both proteins, we can be confident to say that these two proteins were contained in bands 1 and 2 because of the accuracy of the MALDI measure-

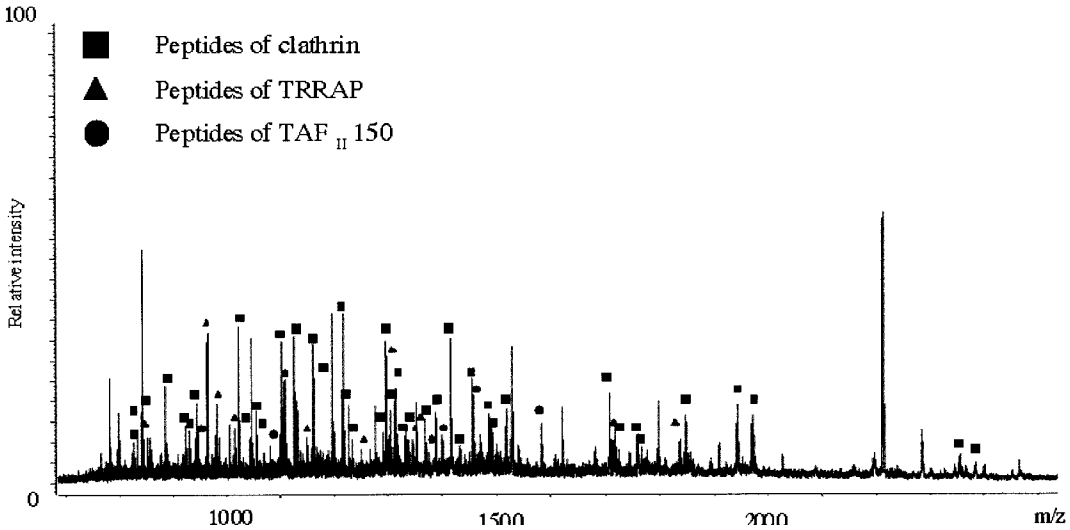

Figure 2. MALDI spectrum of band 2, 107 peaks were measured in the *m/z* 750–2400.

Table 1. Summary of protein identification by 1-D SDS PAGE followed by MALDI-TOF MS analysis

Spot	Protein identity	Sequence coverage	Accession number
1	Clathrin heavy chain	12%	P49951
	TRRAP	3%	Q9Y6H4
2	Clathrin heavy chain	28%	P49951
	TRRAP	5%	Q9Y6H4
	TAFII-150	7%	O43604
3	Clathrin heavy chain	27%	P49951
	TAFII-150	15%	O43604
4	TAFII-135	22%	O00268
	KIA0017/SAP130	15%	Q15393
5	TAFII-100	14%	Q15542
	KIA0017/SAP130	10%	Q15393
6	TAFII-100	11%	Q15542
7	TAFII-100	12%	Q15542
8	TAFII-100	26%	Q15542
	GCN5	15%	P78537
9	TAFII-80	36%	P49848
		21%	Q91857
10	Serum albumin	42%	P07724
	PAF 65α	28%	NP_006464
	PAF 65β	23%	NP_055224
11	PAF 65β	12%	NP_055224
12	SPT3	20%	P49223
13	TAFII-32	44%	Q62880
14	TAFII-20	15%	Q16514

ments and previous identifications using Western blot analysis. Thus our results indicate that it is predominantly the quality of the peptide mass data which determines the quality of the search results. The more accurate the data, the more conclusive are the matches [14, 20, 21].

By using the masses obtained from the different bands by MALDI-TOF MS we identified twelve additional subunits of TFTC: TRRAP, $TAF_{II}150$, $TAF_{II}135$, KIA0017/SAP130, $TAF_{II}100$, GCN5, $TAF_{II}80$, PAF65α, PAF65β, SPT3, $TAF_{II}32$ and $TAF_{II}20$ (Table 1 shows the combined results of the two gels with the identified proteins from SWISS-PROT and NCBInr).

Thus our mass spectrometric identifications confirmed the presence of the majority of the TFTC subunits, which were previously revealed by Western blot and/or Edman based microsequencing analysis. Some of these proteins (*i.e.* $TAF_{II}135$, GCN5, PAF65β, SPT3 and $TAF_{II}20$) have been previously identified exclusively by immune detection methods using subunit specific antibodies [3, 6] and see Table 2. The presence of these proteins in TFTC is now clearly established by an independent method. Together these results indicate that these proteins are indeed *bona fide* subunits of TFTC. In contrast, some of the subunits which were previously identified as TFTC subunits (such as $TAF_{II}30$, $TAF_{II}55$, or ADA3) by microsequencing and/or Western blot analysis were not identified in our study. $TAF_{II}55$ and ADA3 may have been overlooked in our mass determination because in an SDS-PAGE they are not well separated from the heavy chain of the antibodies that contaminated the TFTC fraction (Fig. 1). Thus, $TAF_{II}55$- and ADA3-containing gel pieces may not have been excised from the gel. Similarly $TAF_{II}30$ migrates very close to the light chain of the used antibodies and thus we may have missed it when excising stoichiometric protein species of TFTC.

Table 2. Summary of the TFTC subunits obtained by using different protein identification methods. Comparison between the results obtained by Western blot analysis [3, 6], Edman microsequencing [12] and MS analysis.

Name	Mass (kDa)	Western blot	Micro-sequencing	Mass spectrometry
TRRAP	400	X	X	X
$TAF_{II}150$	150	X	X	X
$TAF_{II}135$	135	X	–	X
SAP130	130	X	X	X
$TAF_{II}100$	100	X	X	X
GCN5	100	X	–	X
$TAF_{II}80$	80	X	X	X
PAF65α	65	N.D.	–	X
PAF65β	65	X	–	X
$TAF_{II}55$	55	X	X	–
ADA3	50–55	X	X	–
SPT3	35	X	–	X
$TAF_{II}32$	32	N.D.	–	X
$TAF_{II}30$	30	X	X	–
$TAF_{II}20/15$	20	X	–	X

X = identification
– = no identification
N.D. = not done because of the lack of high specificity antibodies

3.2 Identification of two novel TFTC subunits

More importantly, two new subunits of the complex were clearly identified, *e.g.* $TAF_{II}32$ and PAF65α as being a part of the TFTC complex. Interestingly, $TAF_{II}32$ and PAF65α are also present in the PCAF/GCN5 complex that shows a very similar polypeptide composition as TFTC [10]. The identification of these two subunits as being part of TFTC is crucial for a better understanding of the structure as

well as the function the complex. Firstly, it has been previously demonstrated that $TAF_{II}80$ (present also in TFTC) can interact *in vitro* with $TAF_{II}32$ through an interaction similar to the one existing between histones pairs. This histone fold interaction domain, which allows an antiparallel "head to tail" interaction between the two partners, notably implicating a hydrophobic interaction between two amphipatic alpha helices, has thus been called "histone fold domain" (HFD). This interaction is thought to play an important role in the stability of the structure of the TFIID complex as well. Indeed the crystal structure of the interaction between the *Drosophila* homologues of human $TAF_{II}32$ and $TAF_{II}80$ has been resolved and showed that these two TAFs interact through a histone fold type domain [22]. Moreover, the importance of this interaction in the $TF_{II}D$ complex have been highlighted more recently by the identification of a specific type of $TF_{II}D$ complex (called $TF_{II}D\pi$) which contains a different isoform of $TAF_{II}80$ mutated in the HFD and which does not contain $TAF_{II}32$ [23].

These results together demonstrated that most probably $TAF_{II}32$ and $TAF_{II}80$ interact in the $TF_{II}D$ complex and that the absence of $TAF_{II}32$ is important at the functional level as the $TF_{II}D\pi$ complex seems to play a very specific role in the mechanism of inducing apoptosis. As the TFTC complex contains a $TAF_{II}80$ isoform with nonmutated HFD [23], we suspected the presence of $TAF_{II}32$ in the TFTC complex, without being able to clearly show it. The present identification of $TAF_{II}32$ as being a subunit of the TFTC complex allowed us to clarify this important point. Moreover, our results suggest that $TAF_{II}80$ and $TAF_{II}32$ probably interact through their HFD inside the TFTC complex and thus participate in its stability and function. This similarity of the interacting partners between the TFTC and $TF_{II}D$ complexes is in accordance with the overall similarity observed between the three dimensional structures of these two complexes reconstituted by electron microscopy and digital image analysis [4].

Interestingly, the other newly identified TFTC subunit, human PAF65α, shows a strong sequence homology to $TAF_{II}80$ and also contains a putative HFD [10]. The presence of this domain in PAF65α has not yet been demonstrated by structural studies, but deduced from sequence comparisons with other histone fold domains. Thus, PAF65α may also interact with $TAF_{II}32$ in the TFTC complex. Unexpectedly, in a recent study we have demonstrated that in each HFD-containing TAF_{II} is present twice, in two distinct lobes of yeast $TF_{II}D$, thus revealing a more complicated molecular organization of $TF_{II}D$ than originally expected [24]. It is thus conceivable that in the TFTC complex $TAF_{II}32$ also participates in interactions with two distinct HFD-containing partners, once with $TAF_{II}80$ and once with PAF65α. Further careful structural analysis of TFTC complex will be necessary to reveal the interaction partners of $TAF_{II}32$. The identification of $TAF_{II}32$ and PAF65α in the TFTC complex has thus opened important new perspectives for the structure and function of this complex.

We have preferred the approach using first a 1-D gel separation of the different proteins present in the purified complex followed by identification of each individual band. This approach is known to be thorough and has been largely demonstrated in the case of protein complexes [25–27] and was preferred to the other possible approach. The 1-D gel followed by MALDI-TOF MS analysis strategy, was selected because it was, in our hands, the more robust one. It is worth pointing out because the sample was particularly difficult to obtain and only a single experiment (apart from the control experiment) was possible.

4 Concluding remarks

Sensitivity is one of the major advantages of the MS approach, compared to microsequencing with 10–20 times less material. Even if the use of 1-D gel allows the possibility of having several proteins in a single band or a minor protein covered by a major one, 1-D gels allow the analysis of hydrophobic proteins, which is not the case with 2-D gels.

The MS approach also permitted a functional analysis of the complex. Other MS based technologies were described earlier where the purified complex is directly digested with trypsin and the tryptic peptides are analysed by LC-MS/MS [28]. Since gel electrophoresis is performed by this technique, the method is particularly fast, but is hampered by the fact that no control is given on the purity of the complex and thus contaminating proteins could be described as a part of a complex. Furthermore, clear information can be obtained on the molecular mass of the analyzed polypeptide, that could be very important when identifying novel or already known polypeptides. Finally, the method described in this study by combining 1-D gel electrophoresis with MALDI-TOF MS ensures a rapid and high fidelity identification of proteins. Moreover, identification of $TAF_{II}32$ and PAF65α in the TFTC complex has opened important new perspectives for the structure/function analysis of this complex.

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Note added in proof: During the processing of this manuscript a new TAF nomenclature has been published. For this new nomenclature see Tora, L., *Genes Devel*. 2002, *16*, 673–675.

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