

Research Article

Studies of recombinant TWA1 reveal constitutive dimerization

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The mammalian muskelin/RanBP9/C-terminal to LisH (CTLH) complex and the *Saccharomyces cerevisiae* glucose-induced degradation (GID) complex are large, multi-protein complexes that each contain a RING E3 ubiquitin ligase. The yeast GID complex acts to degrade a key enzyme of gluconeogenesis, fructose 1,6-bisphosphatase, under conditions of abundant fermentable carbon sources. However, the assembly and functions of the mammalian complex remain poorly understood. A striking feature of these complexes is the presence of multiple proteins that contain contiguous lissencephaly-1 homology (LisH), CTLH and C-terminal CT11-RanBP9 (CRA) domains. TWA1/Gid8, the smallest constituent protein of these complexes, consists only of LisH, CTLH and CRA domains and is highly conserved in eukaryotes. Towards better knowledge of the role of TWA1 in these multi-protein complexes, we established a method for bacterial expression and purification of mouse TWA1 that yields tag-free, recombinant TWA1 in quantities suitable for biophysical and biochemical studies. CD spectroscopy of recombinant TWA1 indicated a predominantly α -helical protein. Gel filtration chromatography, size-exclusion chromatography (SEC) with multi-angle light scattering (SEC-MALS) and native PAGE demonstrated a propensity of untagged TWA1 to form stable dimers and, to a lesser extent, higher order oligomers. TWA1 has a single cysteine residue, Cys¹³⁹, yet the dimeric form was preserved when TWA1 was purified in the presence of the reducing agent tris(2-carboxyethyl)phosphine (TCEP). These findings have implications for understanding the molecular role of TWA1 in the yeast GID complex and related multi-protein E3 ubiquitin ligases identified in other eukaryotes.

Introduction

The muskelin/RanBP9/CTLH complex (referred to subsequently as the MRCTLH complex) is a large multi-protein complex that has been identified in cells from several mammalian species [1,2]. The protein components of this complex include muskelin, RanBP9/RanBPM, TWA1, MAEA, Rmnd5a and Armc8 [1]. Molecular cell biological and gene knockout or knockdown studies of individual proteins indicate a wide repertoire of activities that include roles of muskelin in cell morphology, transcriptional regulation and synaptic processes [3-5]; of RanBP9 in ERK signalling, neurodegeneration and spermatogenesis [6-8]; and of Armc8 in modulating stability of hepatocyte growth factor-regulated tyrosine kinase substrate or β -catenin [9,10]. *Xenopus tropicalis* Rmnd5 has been demonstrated to have E3 ubiquitin ligase activity *in vitro* and to be important for embryonic development of the *Xenopus* mesencephalon, prosencephalon and eye *in vivo* [11]. However, how the specific role(s) of each protein relate to the assembly and function of the complex remains unclear.

A homologous complex in the budding yeast *Saccharomyces cerevisiae* is termed the glucose-induced-degradation deficient (GID) complex and has been studied in detail. The 600 kDa GID complex is a multi-protein E3 ubiquitin ligase that is activated by the association of an additional protein, Gid4, to poly-ubiquitinate gluconeogenic enzymes such as fructose 1,6-bisphosphatase and targets them for proteasomal degradation following a shift of yeast cells from a non-fermentable carbon source

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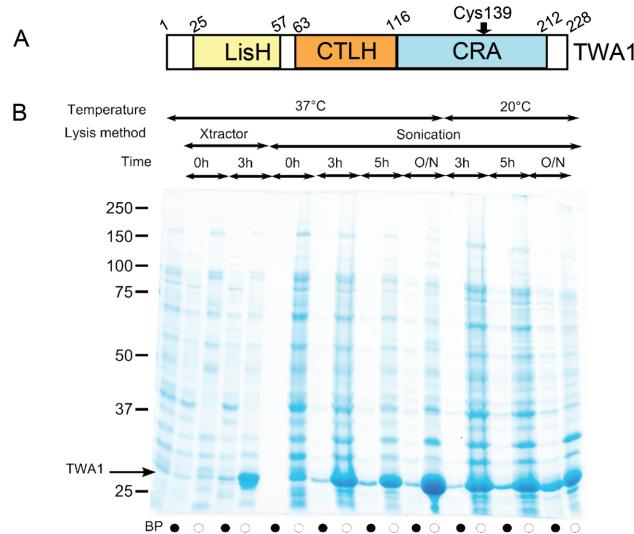


Figure 1. TWA1 and set up of bacterial expression

(A) Schematic diagram of the domain organization of TWA1. Numbers are based on the human and mouse proteins, which have 99% sequence identity. (B) SDS/PAGE analysis of lysate supernatants (black circles) or lysate pellets (white circles) from TWA1.V5His6-expressing bacteria grown at the indicated temperatures, without or with induction with 1 mM IPTG; BP = bacterial pellet of uninduced cells. Lysates were prepared with commercial buffer (Xtractor) or by sonication. Samples were analysed under reducing conditions on SDS/PAGE (12% gel) and the proteins visualized by Coomassie blue staining.

to a fermentable carbon source [12]. The constitutive GID complex includes Gid1, a large multi-domain scaffolding protein (orthologous to RanBPM/RanBP9); an enzymatically active RING E3 ligase Gid2 (orthologous to Rmnd5a); an enzymatically inactive variant RING protein Gid9 (orthologous to MAEA), which dimerizes with Gid2 and is necessary for Gid2 activity; Gid5 (orthologous to Armc8); Gid7, which is bound by Gid4 to activate the complex and a small scaffolding protein Gid8, (orthologous to TWA1) that interacts with several other proteins in the complex [13]. Gid1, Gid4 and Gid5 also function in vacuole-mediated degradation of fructose 1,6-bisphosphatase, a pathway equivalent to metazoan endo-lysosomal protein degradation [14]. RanBPM-binding proteins have been identified in the plant *Arabidopsis thaliana*, which include orthologues of TWA1/Gid8, MAEA/Gid9, Rmnd5/Gid2 as well as an orthologue of mammalian WDR26, which has domains in common with muskelin and Gid7 [15]. The Rmnd5/Gid2 orthologue of the plant *Lotus japonicus* has E3 ligase activity *in vitro* [16]. Collectively, these observations implicate E3 ligase activity as a conserved property of these distinct yet related multi-protein complexes identified in different lineages of eukaryotes.

We characterized previously *in silico* that many of the protein components of the GID and MRCTLH complexes are present in multiple lineages of extant eukaryotes, indicative of an origin in the last eukaryotic common ancestor [17]. Another striking feature of the MRCTLH, GID and *Arabidopsis* complexes is the related domain composition of many of the constituent proteins. RanBP9/Gid1, Rmnd5/Gid2, TWA1/Gid8 and MAEA/Gid9 are all multi-domain proteins that include, in the same domain order, a lissencephaly-1 homology (LisH) domain, a C-terminal to LisH (CTLH) domain and a C-terminal CT11-RanBP9 (CRA) domain. The LisH and CTLH domains are both characterized as largely α -helical regions. A conserved feature of LisH is the presence of N-terminal and C-terminal α -helices connected by an apical loop [17]. In lissencephaly-1 (Lis1), LisH mediates assembly of anti-parallel dimers [18]. The CTLH domain was identified as an α -helical region frequently found C-terminal to a LisH domain [19]. CTLH domains are predicted to contain three α -helices, separated by short loops [17]. The CRA domain is a region of approximately 100 amino acids that includes six α -helices [17,20]. These three domains comprise the entirety of TWA1/Gid8 (Figure 1A), whereas RanBP9, Rmnd5 or MAEA each contain additional domains [13,17]. Muskelin, which is not encoded in *S. cerevisiae*, is a RanBP9-binding protein in the MRCTLH complex and also contains LisH and CTLH domains, as do Gid7 and WDR26 [3,12,15]. These shared attributes of domain architecture suggest that LisH and CTLH domains could be important for the assembly and function of the complex. In general, LisH domains participate in protein oligomerization by the formation of anti-parallel dimers [21–26]. However, there are also indications that the LisH domains of MRCTLH proteins can have distinct functions. The LisH domain of muskelin includes a

cryptic nuclear localization sequence that is likely to be activated by phosphorylation of a conserved threonine residue adjacent to the C-terminus of muskelin [3]. Sequence analyses of the LisH and CTLH domains of MRCTLH proteins have indicated that the LisH and CTLH domains of TWA1 and RanBP9 have related conserved features, whereas these domains in MAEA and Rmnd5 have different profiles of conserved residues [17].

For further investigation of the structural/functional relationships of the LisH, CTLH and CRA domain organization, TWA1/Gid8 is of particular interest because it is the smallest protein within the complex that has LisH, CTLH and CRA domains (Figure 1A), because of its presence in all forms of this ubiquitin E3 ligase complex identified to date and because of its very high conservation across eukaryotes [1,2,12,15,17]. However, relatively little is known about the functional roles of either TWA1 or Gid8. TWA1 is a direct binding partner of RanBP9 [2] and is present in both the nucleus and cytoplasm of mammalian cells [17]. Similarly, Gid8 interacts directly with Gid1 [13]; in addition, *gid8* deletion mutants in *S. cerevisiae* indicated that Gid8 functions as an adapter for Gid2 and Gid9 [13]. An *in silico* prediction of protein–protein domain interactions in *S. cerevisiae* implicated a strong involvement of Gid8 within a “core” GID complex: tandem affinity purification of Gid1 and the loss of several Gid1-co-purifying proteins in a *Gid8* gene deletion yeast strain (Δ YMR135C) give substance to this hypothesis [27]. Whereas LisH domains from several unrelated proteins have been crystallized [18,24,28] and the LisH domain of muskelin has been crystallized in combination with its N-terminal discoidin domain [29], almost nothing is known about the biochemical properties of TWA1, Gid8 or their orthologues in other eukaryotes. In transient expression experiments in mammalian COS-7 cells, we noted that TWA1 could be expressed to higher levels than the RING domain-containing proteins, Rmnd5 or MAEA [17]. Given the potential importance of LisH, CTLH and CRA domains for the activities of multiple proteins within the MRCTLH or GID complexes, we established a system for bacterial expression and purification of TWA1 and report here on a biochemical study of the purified TWA1 protein.

Materials and methods

Molecular biology procedures

TWA1 cDNA (from mouse, 99% amino acid sequence identity to human TWA1) was amplified by PCR from a previously described plasmid [17], with DNA oligonucleotide primers 703F (5'-CACCATGAGTTATGCAGAAAAACCCG-3') and 268R (5'-GATCGAATTCGCTCTACTTGGGCTCCTC-3') (Sigma-Aldrich, U.K.) and ligated into pET151/D-TOPO bacterial expression plasmid (Invitrogen) following the manufacturer's instructions. This plasmid supplies an N-terminal V5His6 tag with a following Tobacco Etch Virus nuclear inclusion α endopeptidase protease (TEV) cleavage site to the encoded protein.

Pilot experiments for TWA1 expression in *Escherichia coli*

Unless otherwise stated, chemicals were from Sigma-Aldrich. IPTG induction of recombinant TWA1.V5His6 expression in *E. coli* BL21(DE3) cells (Invitrogen) was performed on 5 ml cultures to elucidate appropriate conditions for TWA1.V5His6 expression. Single *E. coli* colonies were inoculated into LB containing 100 μ g/ml ampicillin (LB/amp) and grown overnight at 37°C with shaking. The next day, 1 ml of each culture was used to inoculate 9 ml of fresh LB/amp medium. Cultures were incubated at 37°C until an A_{600} between 0.4 and 0.6 was reached. Then, 500 μ l of each culture was collected, centrifuged at 13000 rpm for 2 min and the pellet was stored at -20°C as the ‘0h’ sample. The remaining culture was split into two equal portions. Expression of TWA1.V5His6 was induced in one of these by the addition of IPTG to a final concentration of 1 mM. Growth was continued at 37°C and 1 ml of each culture was collected at designated times up to 16 h (overnight). At each timepoint, the bacteria were pelleted by centrifugation and stored at -20°C. In a parallel experiment, the cultures were grown at 20°C after IPTG induction. To compare the extractability of TWA1.V5His6 using different methods of bacterial cell lysis, bacterial pellets corresponding to 0 h or 3 h timepoints were either lysed by gentle agitation in 500 μ l of Xtractor buffer (Clontech) at room temperature for 1 h or resuspended in 1 ml of lysis buffer (200 mM NaCl, 50 mM TRIS, pH 8.0, 10% glycerol, 25 mM imidazole and 2 mM PMSF) and disrupted by sonication for 2 min (four cycles of 5 s bursts at 25 s intervals) on ice. All lysed samples were centrifuged at 18000 g for 30 min at 4°C to separate cell supernatants from residual pellets. Pellets were resuspended in 100 μ l 2× SDS/PAGE sample buffer containing 100 mM DTT and supernatants were mixed 1:1 with the same buffer. Thirty microlitres of each sample were resolved on SDS/PAGE (10% gel) under reducing conditions. Proteins were visualized on gels by Coomassie blue-based staining with Gelcode® Blue (Thermo Fisher).

Expression and purification of TWA1.V5His6

For expression of TWA1.V5His6 at larger scale, a single colony from LB-ampicillin plates streaked with *E. coli* BL21(DE3) cells transformed with pET151-/D-TOPO:TWA1.V5His6 was added to 10 ml LB/amp medium and the

culture grown overnight at 37°C. This culture was then transferred into 1 litre of fresh LB/amp medium and grown at 37°C until an A_{600} of 0.6 was reached. IPTG was added to a final concentration of 1 mM, and cultures were grown for 5 h at 18–20°C, harvested by centrifugation (10000 $\times g$ for 30 min at 4°C) and resuspended in 40 ml of cold lysis buffer consisting of 200 mM NaCl, 50 mM TRIS, pH 8.0, 10% glycerol, containing 25 mM imidazole and 2 mM PMSF. Cells were disrupted by sonication for 8 min (eight cycles of 15 s bursts at 45 s intervals) on ice and centrifuged at 18000 $\times g$ for 30 min at 4°C. The cell supernatant was applied to a His-trap FF column (GE Healthcare), after stripping with EDTA and reloading with NiSO₄, connected to an Akta System (GE Healthcare) and allowed to flow through, resulting in binding of TWA1.V5His6 to the column. After binding, lysis buffer and elution buffer (200 mM NaCl, 50 mM TRIS, pH 8.0, 10% glycerol, containing 1 M imidazole and 2 mM PMSF) were applied to the column to establish a 25 mM to 1 M imidazole gradient. Protein elution was monitored by protein UV absorbance detection at 280 nm, and the eluent was collected as 1 ml fractions. The purity of the protein preparation in each fraction was assessed by SDS/PAGE using NuSEP TRIS/glycine gels (Generon). Fractions containing TWA1.V5His6 were pooled and dialyzed against 50 mM EDTA, 1 mM TRIS overnight. The following day, 25 µg of TEV protease (recombinant protein, the gift from Skye Hodson and Steven Burston, University of Bristol) was added to the pooled fractions to cleave off the N-terminal tags. This was achieved by dialysing the mixture against fresh TEV buffer overnight. The mixture was then loaded on a 5 ml HisTrap FF column to bind the His6-tagged protease, yielding cleaved, tag-free TWA1 in the flow through. Flow-through fractions were assessed for protein purity on NuSEP TRIS-glycine gels. The peak TWA1 containing fractions were pooled and concentrated to 2–3 ml by ultrafiltration through a 10 kDa cut-off membrane (Amicon Ultra-15 Millipore). TWA1 was further purified by gel filtration on a HiLoad 16/600 Superdex 75 pg column, with column bed volume of 120 ml (GE Healthcare), in a buffer of 200 mM NaCl, 50 mM TRIS, pH 8.0. Eluted fractions were assessed for purity by SDS/PAGE. The TWA1-positive fractions were pooled and concentrated by ultrafiltration through a 10 kDa pore membrane to a concentration of 10 mg/ml and stored at 4°C for subsequent experiments. Molecular weight standards applied to the same column included alcohol dehydrogenase (80 kDa), HLA-A2/β2-microglobulin (46 kDa), CD8a (30 kDa) and β2-microglobulin (11 kDa). Protein concentrations were measured with a NanoDrop Lite spectrophotometer (Labtech).

Circular dichroism

CD spectroscopy measurements were conducted using a JASCO J-810 spectropolarimeter fitted with a Peltier temperature controller (Jasco, U.K.). Purified recombinant TWA1 was prepared at 5 µM in PBS. Full CD spectra were recorded from 240 nm to 190 nm in 0.1 mm path-length quartz cuvettes at 5°C. The instrument was set with a scan rate of 100 nm/min, a 1 nm bandwidth and a 1 s integration time. Scans were retrieved and data analysis was carried out with the CONTIN deconvolution algorithm [30] at the DichroWeb server [31] using protein reference set 4 [32].

Size-exclusion chromatography with multi-angle light scattering

Size-exclusion chromatography (SEC) with multi-angle light scattering (SEC-MALS) was performed at room temperature. A sample (500 µl) of purified TWA1 at a concentration of 2 mg/ml was injected on to an S200 300/10 GL analytical size-exclusion column (GE Healthcare) attached to a light scattering diode array (Dawn Heleos II, Wyatt Technology, U.K.) and a differential refractive index detector (Optilab rEX, Wyatt Technology, U.K.). The column was pre-equilibrated at room temperature with buffer containing 50 mM TRIS, 200 mM NaCl and 0.5 mM tris(2-carboxyethyl)phosphine (TCEP). Detector normalization was achieved by the use of BSA (Sigma-Aldrich).

SDS/PAGE

To denature proteins for PAGE, samples were boiled for 10 min in SDS/PAGE sample buffer. In some experiments, samples with a final volume of 30 µl were separated on 10% (w/v) polyacrylamide gels under reducing conditions. In other experiments, samples with a final volume of 10 µl were loaded on NuSEP TRIS-glycine gels. Electrophoresis with handcast gels was performed in a GIBCO BRL Vertical Gel Electrophoresis apparatus with running buffer of 0.2 M glycine, 0.025 M TRIS and 0.1% SDS at 45 V. Electrophoresis with precast NuSEP gels was performed in a Mini Protein II gel apparatus (Bio-Rad) with TRIS-glycine running buffer at 130 V.

Native PAGE

Samples were mixed 1:1 with native PAGE gel loading buffer (final concentrations, 87 mM TRIS, 0.2% bromophenol blue and 0.004% glycerol, containing maximum of 1 mM DTT) immediately prior to loading on to 10% native polyacrylamide gels. The resolving gel contained 375 mM TRIS, pH 8.8. The stacking gel contained 625 mM TRIS, pH 6.8. Electrophoresis was performed with native PAGE running buffer (0.19 M glycine, 25 mM TRIS, pH 8.8) at 15V.

Western blotting

After resolution by SDS/PAGE, proteins were transferred electrophoretically to 0.22 µm pore PVDF membrane (Millipore) by semi-dry blotting (Bio-Rad), in transfer buffer consisting of 0.19 M glycine, 25 mM TRIS, pH 7.1 and 20% methanol. Membranes were blocked for 1 h in Western blot blocking buffer (2% [w/v] powdered milk in 1× TRIS buffered saline at pH 7.5 containing 0.2% Tween 20) and then incubated in a sealed polythene bag with mouse monoclonal antibody to V5 tag (1:2500; Clontech), in 12 ml of Western blot blocking buffer for 3 h at room temperature with rotation at 350 rpm. Membranes were then washed three times in 100 ml of fresh blot blocking buffer, for 5 min each wash, with rotation at 100 rpm. Membranes were then incubated in a sealed bag with goat anti-mouse IgG conjugated to alkaline phosphatase (1:5000; Life Technologies) in 10 ml fresh Western blot blocking buffer for 1 h at room temperature with rotation at 350 rpm. Membranes were washed three times as described above, rinsed twice in TRIS-buffered saline and developed in 6 ml of Western blot assay buffer (1 mM MgCl₂ in 1% (v/v) diethanolamine containing 60 µl chloro-5-substituted adamantyl-1,2-dioxetane phosphate [CSPD; Life Technologies]) for 5 min. Membranes were covered in a polythene sleeve and exposed to ECL Hyperfilm (Amersham) that was developed in a CURIX 60 (AFGA) X-ray processor.

Mass spectrometry

Protein bands were cut into 1 mm² pieces and digested with sequencing grade trypsin (Promega) using a ProGest automated digestion unit (Digilab Ltd.). The resulting peptides were analysed by MS. Mass spectra were recorded in positive ion reflector mode on an Applied Biosystems 4700 MALDI mass spectrometer. For MS/MS analysis, the top five most intense, non-tryptic precursors were selected for fragmentation by collision-induced dissociation. Neither baseline subtraction nor smoothing was applied to recorded spectra. MS and MS/MS data were analysed using GPS Explorer 3.5 (Applied Biosystems). MS peaks were selected between 800 and 4000 Da and filtered with a minimum signal-to-noise ratio of 15 and to exclude masses derived from trypsin autolysis. MS/MS peaks were filtered to exclude peaks with a signal-to-noise ratio less than 10 over a mass range of 50 Da to 20 Da below the precursor mass.

Data were analysed using the MASCOT algorithm (Matrix Science) and searched against the NCBI Human protein database. A maximum number of missed cleavages of 1 and a charge state of +1 were assumed for precursor ions. Peptide precursor mass tolerance was set at 100 ppm, and MS/MS tolerance was set at 0.25 Da. Search criteria included carbamidomethylation of cysteine as a fixed modification and oxidation of methionine as a variable modification. A MASCOT score greater than 64 (the default MASCOT threshold for such searches), corresponds to a statistically significant ($P < 0.05$), confident identification. TWA1 alone was recovered from the sample with a MASCOT score >65 (See Supplementary Data).

Results and discussion

Bacterial expression of TWA1.V5His6

The bacterial expression plasmid used, pET151/D-TOP, supplies an N-terminal V5 and His6 tag with a following TEV cleavage site. Pilot experiments were carried out with 5 ml *E. coli* cultures (as described in the Materials and methods section) to determine conditions suitable for IPTG-induced expression of TWA1.V5His6. Cell lysates were prepared at several timepoints, either with a commercial extraction buffer (Xtractor) or by sonication of bacterial suspensions. The supernatant and pellet fractions were analysed by SDS/PAGE after reduction by DTT. A strong band of TWA1.V5His6 was present in the pellet of induced, Xtractor-lysed cells after 3 h of induction at 37°C. This protein resolved with an apparent molecular mass of 33 kDa, consistent with the predicted molecular mass of TWA1 plus the V5 and 6-His tags (Figure 1B, lane 5). However, the supernatant fraction contained little TWA1.V5His6 (Figure 1B, lane 4). TWA1.V5His6 was also detected in the pellets of sonicated cells after 3h, 5h or 16h of IPTG-induction at 37°C (Figure 1B) and small amounts of TWA1.V5His6 were detected in supernatants from sonicated cells at timepoints beyond 3 h (Figure 1B). Combining growth of *E. coli* at 20°C during the induction period with lysis by sonication increased the amount of TWA1.V5His6 in the supernatant fraction, after either 5 h or overnight induction times (Figure 1). As a compromise between optimizing protein folding conditions (favoured by lower temperature and tapered growth period) and maximizing protein yield (favoured by the most effective lysis method and maximal growth period), 5 h was chosen as the IPTG induction period, 20°C was chosen as the growth temperature during IPTG induction and cell lysis by sonication was chosen as the extraction method for larger-scale purifications of TWA1.V5His6.

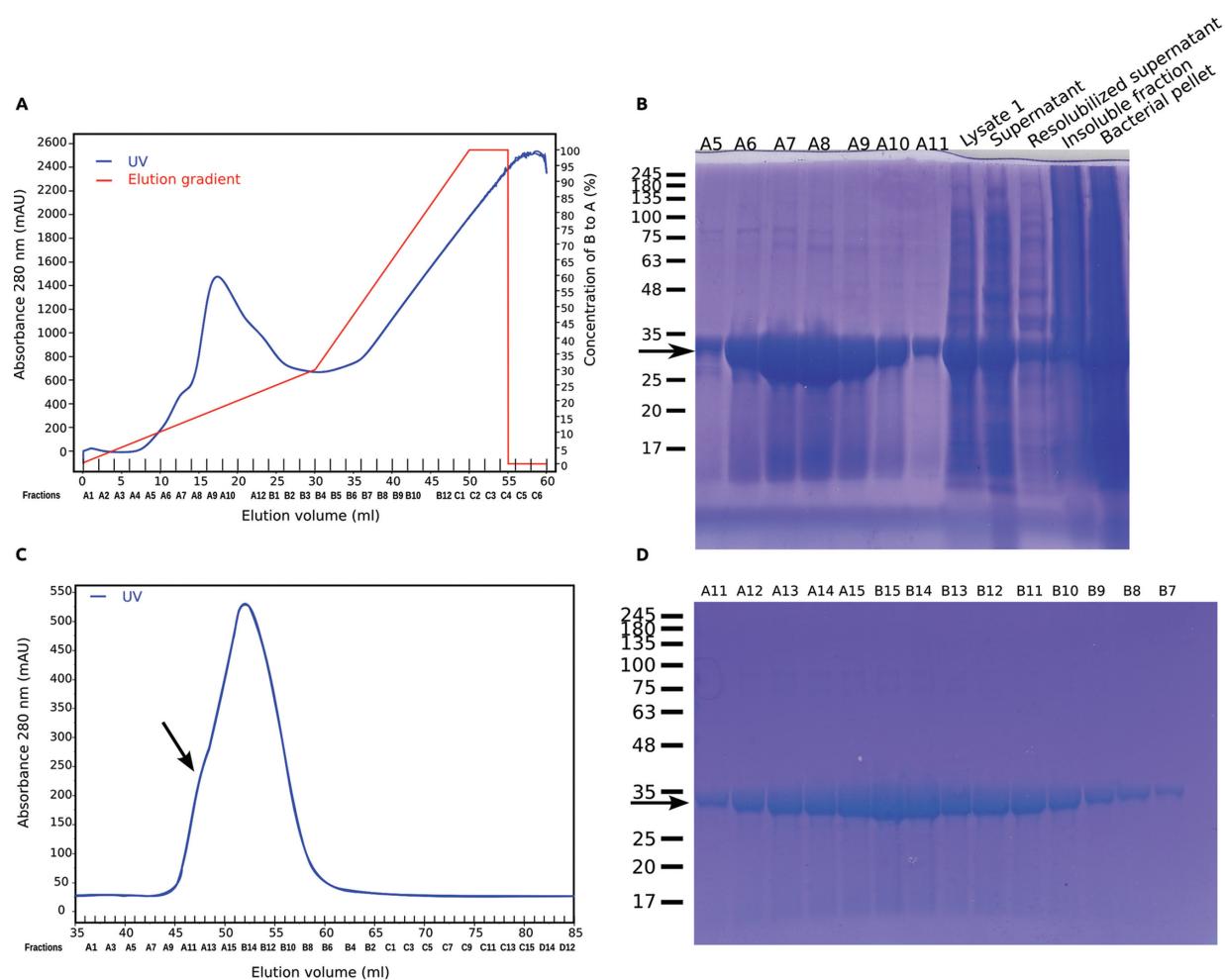


Figure 2. Purification of bacterially expressed TWA1.V5His6

(A) UV trace from metal affinity chromatography of lysates of sonicated *E. coli* containing TWA1.V5His6. (B) SDS/PAGE analysis of equal volumes (30 µl) of fractions A5–A11 across the main protein peak from the metal affinity chromatography along with samples of the total bacterial pellet (“pellet”), initial lysate (“Lysate”) and supernatant after centrifugation (“supernatant”). For comparison, supernatant “resolubilized supernatant” and pellet (“insoluble pellet”) after 8 M urea extraction are shown. Arrow indicates TWA1.V5His6. (C) UV trace from SEC of the pooled peak TWA1.V5His6 fractions from the metal affinity chromatography step. Arrow indicates the shoulder peak. (D) SDS/PAGE analysis of equal volumes of fractions across the protein peak from the SEC. Arrow indicates TWA1.V5His6. In (B) and (D), molecular mass markers are given in kDa.

Purification of TWA1.V5His6

With use of larger scale *E. coli* liquid cultures, TWA1.V5His6 was prepared and purified from the cell lysate supernatant of sonicated *E. coli* by nickel affinity chromatography and elution under a 25 mM to 1 M gradient of imidazole. The major peak of protein eluted between 0.186 M and 0.206 M imidazole (Figure 2A). When fractions around the protein peak, along with samples from earlier steps of the protocol, were analysed on TRIS-glycine gels under reducing conditions, a strong band of approximately 33 kDa was apparent with peak protein between fractions A6 and A10 (arrowed in Figure 2B). This protein was identified as TWA1 by MALDI MS analysis of the band from fraction A9 (as in Figure 2A; see also Supplementary Data).

The TRIS-glycine gel analysis also indicated that the fractions that contained the most TWA1.V5His6 also included small amounts of higher molecular mass bands (Figure 2B). Therefore, the peak TWA1.V5His6 fractions from the metal affinity chromatography step were pooled together and fractionated by SEC. The analysis yielded a single major protein peak (Figure 2C). When fractions across the peak were analysed by TRIS-glycine gel electrophoresis under reducing conditions, all fractions contained a major, monodisperse protein band that resolved with a relative

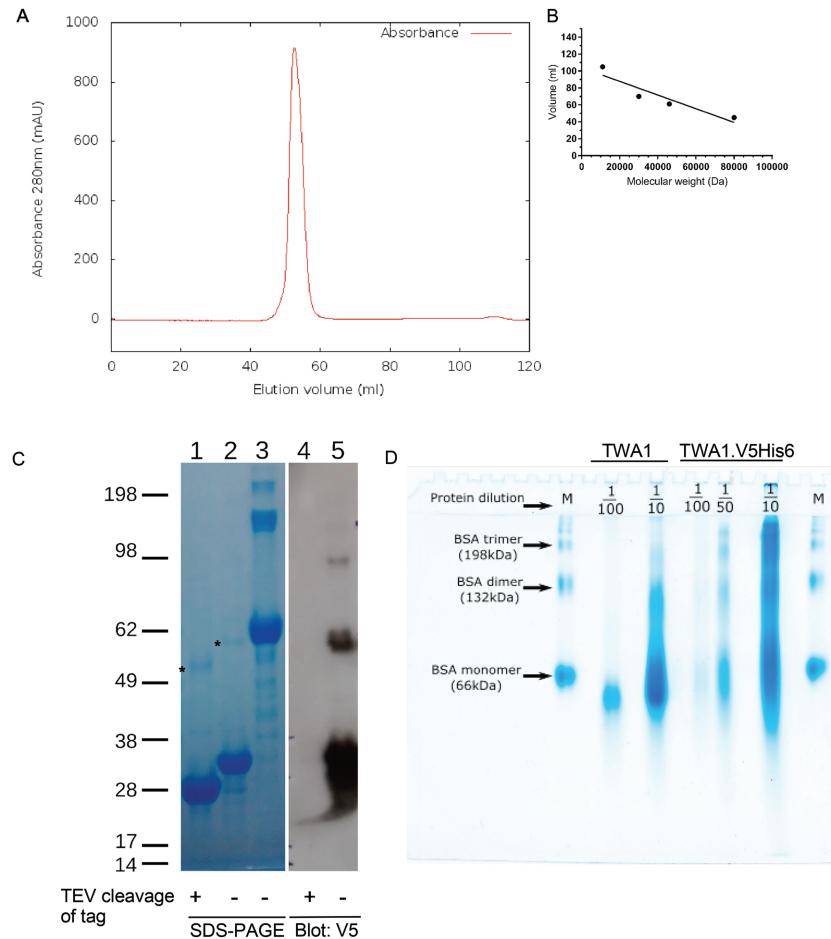


Figure 3. Evidence for thiol-independent dimerization of TWA1

(A) UV trace from SEC of TWA1 purified in the presence of TCEP and removal of the V5His6 tag by TEV protease. (B) Calibration curve of elution volumes of molecular mass standards from SEC. (C) PAGE analysis by reducing conditions on a TRIS-glycine gel of purified, recombinant TWA1 before and after removal of the V5His6 tag by TEV protease. Lanes 1–3: Coomassie blue stained gel. Lanes 4 and 5: immunoblot of replicate lanes on the same gel with V5 antibody. Lanes 1 and 4, TWA1 after tag removal; lanes 2 and 5, TWA1.V5His6; lane 3, BSA. Six micrograms of TWA1 were loaded per lane. Molecular mass markers are given in kDa. (D) Native PAGE separation of purified TWA1 samples, without or with removal of His6 tag. The TWA1 preparations were diluted as indicated to achieve different protein loads/lane. BSA was used as a size marker (M). Electrophoresis was carried out on 8% polyacrylamide gels and proteins visualized by Coomassie blue staining.

molecular mass of approximately 33 kDa and corresponded to TWA1.V5His6 (arrowed in Figure 2D). Yields were approximately 20 mg/l.

The absorbance traces from the SEC also indicated a shoulder peak (arrowed in Figure 2C). To investigate the possibility that TWA1.V5His6 tag might undergo artifactual aggregation, the purification strategy was modified in two ways. Firstly, purifications of TWA1 were carried out in which pooled TWA1 containing fractions from the nickel affinity chromatography step were incubated with TEV to cleave off the portion of the polypeptide containing the N-terminal V5 and His6 tags. The protein was then re-run through the nickel affinity column to retain the V5His6 tag fragment. Tag-free TWA1 was recovered in the flow through. Secondly, TWA1 has a single cysteine residue (Cys¹³⁹) and it is possible that intermolecular thiol–thiol interactions of Cys¹³⁹ could be responsible for dimerization of TWA1. Therefore, the reducing agent TCEP was included in all buffers to maintain thiol groups in a reduced state throughout all purification steps. TCEP was chosen over DTT, a widely used and less costly reducing agent, because TCEP is compatible with most metal affinity matrices and is a more stable reducing agent that can be used at lower concentrations [33]. Analysis of the final purified material by SEC demonstrated a single major protein peak with no shoulder (Figure 3A). In relation to the elution volumes of a set of molecular mass standards (Figure 3B), TWA1

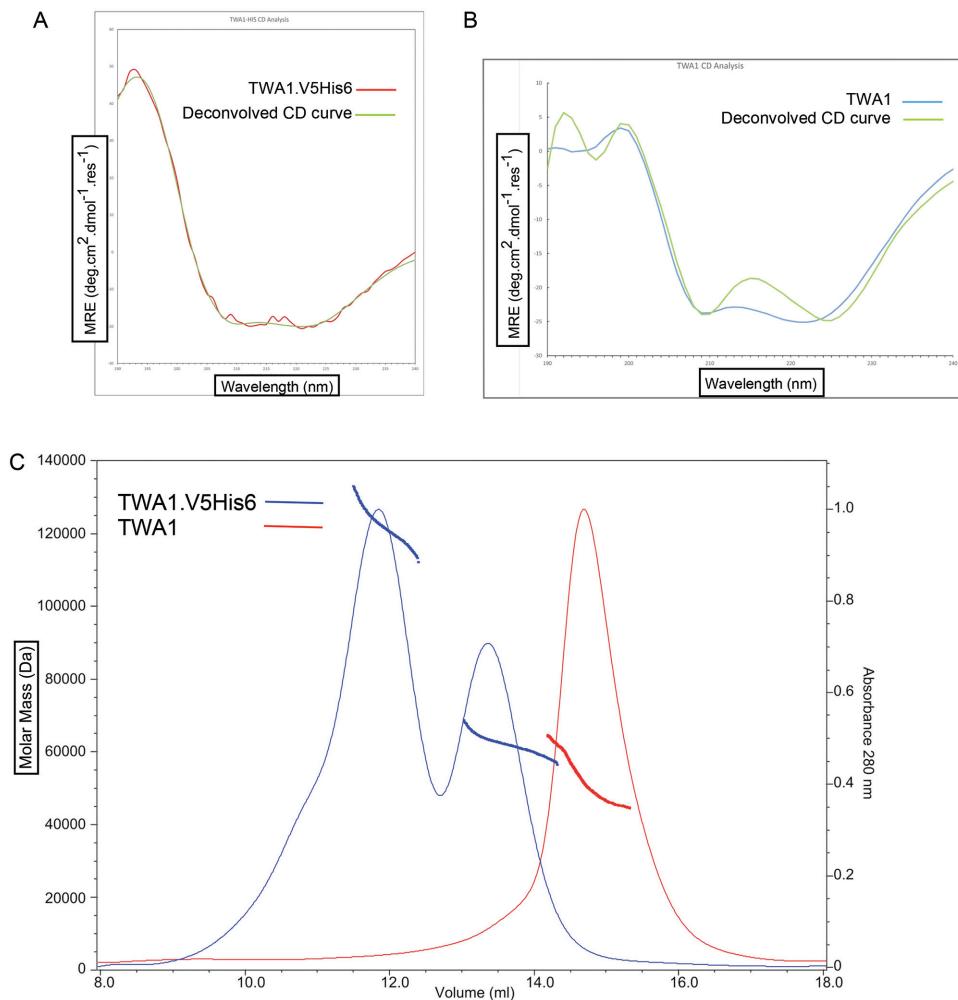


Figure 4. Circular dichroism and SEC-MALS analysis of TWA1

(A) CD spectrum of TWA1.V5His6 (from 8 scans). (B) CD spectrum of tag-free TWA1 (from 32 scans). Both (A) and (B) also include a superimposed, deconvolved CD curve using Contin-4 and -7 reference sets (dichroweb.cryst.bbk.ac.uk). (C) SEC-MALS chromatograms of the elution of TWA1.V5His6 or tag-free TWA1 from a S200 10/300 GL analytical column. SEC MALS analysis of TWA1 before, (blue) and after, (red), cleavage of the V5His6 tag.

eluted with an apparent molecular mass of around 60 kDa. Effective removal of the tags by TEV was confirmed by examination of TWA1.V5His6 compared with TWA1 after TEV cleavage and V5His6-tag fragment removal by reducing SDS/PAGE and immunoblotting. BSA was also run on the gel for additional corroboration of apparent molecular masses (Figure 3C, lane 3). Uncleaved TWA1.V5His6 was detected on the SDS/PAGE gel as a major 33 kDa band and a minor band of approximately 60 kDa (Figure 3C, lane 2, upper band marked with asterisk). These identifications were confirmed by detection of the same bands by immunoblotting with antibody to the V5 tag. An additional V5-positive minor band of approximately 98 kDa was also detected on the blot (Figure 3C, lane 5). After cleavage of the tags, the major TWA1 band on the SDS/PAGE gel had an apparent molecular mass of 28 kDa and the molecular mass of the minor approximately 60 kDa band was correspondingly reduced (Figure 3C, lane 1, upper band marked with asterisk). Neither species was detected on immunoblot by the antibody to the V5 tag, confirming the specificity of the antibody and the effectiveness of the TEV reaction (Figure 3C, lane 4). Interestingly, the persistence of the approximately 60 kDa species after removal of the V5His6 tag and purification in the presence of TCEP implicated the possibility of an unusually stable and thiol-independent mechanism of dimerization. Additional evidence for this possibility was obtained from analysis of TWA1 on native PAGE gels under non-reducing conditions. To assist visualization and interpretation, several different protein loads were analysed for each sample. TWA1.V5His6 ran at a range of molecular masses, suggestive that the tagged protein did have a tendency to aggregate. In contrast, the

predominant species of tag-free TWA migrated close to the reference BSA monomer, with an apparent molecular mass of approximately 60 kDa. No monomeric (33 kDa) TWA1 was detected on these gels (Figure 3D). These results provided further indication that TWA1 forms stable dimers.

Biochemical analysis of recombinant TWA1

Next, the biophysical properties of TWA1.V5His6 and tag-free TWA1 were investigated. TWA1 is predicted to be mostly α -helical [17]. The secondary structure of recombinant TWA1.V5His6 or TWA1 was assessed by CD spectroscopy. The CD spectra obtained with TWA1.V5His6 showed minima at 208 nm and 222 nm, indicative of a predominantly α -helical structure and also had a good fit with the predicted CD trace of an entirely α -helical protein (Figure 4A). Similarly, the CD spectra obtained with tag-free TWA1 had all the characteristics of an α -helical protein (Figure 4B).

To investigate the oligomeric state of recombinant TWA1.V5His6 or TWA1 in solution, SEC-MALS analysis was carried out. For TWA1.V5His6, the largest peak, representing the earliest eluted protein, corresponded to a mass of 122 kDa, as averaged across the peak. A smaller peak, representing later eluted protein, corresponded to an average mass of approximately 63 kDa (Figure 4C, blue line trace). These masses are consistent with tetrameric and dimeric forms of TWA1.V5His6 respectively. For tag-free TWA1, the single peak of later eluted proteins corresponded to an average molecular mass of 52 kDa (Figure 4C, red line trace). This mass is most consistent with dimeric TWA1. The upward slope in the molecular mass range raises the possibility that TWA1 is in equilibrium between different oligomeric states.

Taken together, these data indicate that purified TWA1 undergoes constitutive dimerization in solution. Dimer assembly does not depend on thiol–thiol interactions. In view that the LisH domain has known roles in the dimerization of other proteins such as Lis1 [21], we suspect that the LisH domain has a role in TWA1 dimerization. Indeed, residues that are important in the LisH domain of Lis1, DCAF and FOP1 for dimerization [21–23,28] are conserved strongly in TWA1 orthologues [17]. A recent crystal structure of the LisH and CTLH containing transcriptional repressor, TPL2, also indicates that the LisH domain is involved in dimerization [34]. We suspect that the LisH domain, possibly in cooperation with the CTLH region that also has α -helical characteristics [17,20], may mediate dimerization of TWA1.

In conclusion, we report a method for bacterial expression and purification of TWA1 in which TWA1 can be prepared and rendered tag-free in quantities suitable for biophysical and biochemical studies. The initial biochemical analyses reported here demonstrate constitutive, thiol-independent dimerization of recombinant TWA1. This finding has implications for further analysis of the central role of TWA1 as a highly conserved component in the multi-protein E3 ubiquitin ligases of the yeast GID complex, the metazoan muskelin/RanBP9/CTLH complex and related complexes of other eukaryotes. It will be of future interest to identify the dimerization interface and generate mutants impaired for dimerization through further biophysical and structural studies of recombinant TWA1.

Supporting data

The data set(s) supporting the results of this article in the Supplementary Data.

Acknowledgements

We thank Leo Brady and his laboratory for advice and discussions. We thank Chris Arthur (Chemistry, UoB) and Kate Heesom (Proteomics Facility, UoB) for the proteomics analysis of recombinant TWA1. We thank Craig T. Armstrong for the CD analysis and contribution to CD data interpretation, Dek Woolfson's laboratory and the Peptide Biophysical Facility for access to the Jasco CD spectropolarimeters and Skye Hodson and Steven Burston for the recombinant TEV protease.

Competing interests

The authors declare that they have no competing interests.

Funding

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Author contribution

O.F. conceived and designed the study, carried out experiments, analysed data, prepared figures and wrote the first draft of the manuscript. G.E.B. and P.R.R. conducted SEC-MALS and analysed data. J.C.A. conceived the study, analysed data and wrote the manuscript with O.F. All authors read and approved the final version of the manuscript.

Abbreviations

CRA, C-terminal CT11-RanBP9; CTLH, C-terminal to LisH; DCAF, DDB1 and CUL4 associated factor; FOP, FGFR1 Onco-gene Partner; GID, glucose-induce-degradation deficient; Lis1, lissencephaly-1; LisH, lissencephaly-1 homology; MAEA, macrophage-erythrocyte attachment protein; RING, Really Interesting New Gene domain; SEC, size-exclusion chromatography; SEC-MALS, size-exclusion chromatography with multi-angle light scattering; TCEP, tris(2-carboxyethyl)phosphine; TEV, Tobacco Etch Virus nuclear inclusion α endopeptidase protease; TWA1, two hybrid associated protein 1 (aka chromosome 20 open reading frame 11); WDR, WD repeat domain containing.

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Analysis Information

Report Type	Protein-Peptide Summary by Spot	Analysis Type	Combined (MS+MS/MS)
Sample Set Name	121107	Database	NCBI_human
Analysis Name	human	Creation Date	11/09/2012 13:19:37
Reported By	11/12/2012 11:01:09 - admin	Last Modified	11/09/2012 13:27:18
MS Acq. : Proc. Methods	(Unspecified) : (Unspecified)		
Interpretation Method	(Unspecified)		

Gel Idx/Pos	28/B3	Instr./Gel Origin	ABI4700/121108	Process Status	Analysis Succeeded
Plate [#] Name	[1] 001100002098	Instrument Sample Name		Spectra	11

Rank	Protein Name	Species	Accession No.	Protein Score	Protein Score C. I. %	Protein MW	Protein PI	Pep. Molecular Count	Molecular Function	Biological Process
1	chromosome 20 open reading frame 11, isoform CRA_a [Homo sapiens]		gi 119595724	586	100	26788.7	4.92	14		
	Protein Group									
	RecName: Full=Protein C20orf11; AltName: Full=Two hybrid-associated protein 1 with RanBPM; Short=Twa1		gi 28201788			26788.7	4.9200			
							000762			
							9395			
	chromosome 20 open reading frame 11 [Homo sapiens]		gi 10185083			26788.7	4.9200			
							000762			
							9395			
	chromosome 20 open reading frame 11, isoform CRA_a [Homo sapiens]		gi 119595725			26788.7	4.9200			
							000762			
							9395			
	protein C20orf11 [Homo sapiens]		gi 8923557			26788.7	4.9200			
							000762			
							9395			
	unnamed protein product [Homo sapiens]		gi 7020818			26788.7	4.9200			
							000762			
							9395			

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result Type
993.5588	993.6671	0.1083	109	19	26	LNNLHVQR				Mascot
1427.7603	1427.8761	0.1158	81	32	43	LIMNYLVTEGFK				Mascot
1470.7952	1470.8982	0.103	70	198	209	LLLWAQNELDQK				Mascot

1598.89	1599.0055	0.1155	72	198	210	LLLWAQNELDQKK					Mascot
1598.89	1599.0055	0.1155	72	198	210	LLLWAQNELDQKK	17	0			Mascot
1821.8766	1821.9969	0.1203	66	172	186	VWSEVNQAVLDYENR	108	100			Mascot
1821.8766	1821.9969	0.1203	66	172	186	VWSEVNQAVLDYENR					Mascot
1919.8903	1920.014	0.1237	64	51	67	MESGIEPSVDLETLDER					Mascot
1919.8903	1920.014	0.1237	64	51	67	MESGIEPSVDLETLDER	41	99.313			Mascot
1956.0146	1956.1411	0.1265	65	32	48	LIMNYLVTEGFKEAAEK					Mascot
2078.03	2078.1665	0.1365	66	170	186	QKVVSEVNQAVLDYENR					Mascot
2078.03	2078.1665	0.1365	66	170	186	QKVVSEVNQAVLDYENR	80	100			Mascot
2114.1545	2114.2854	0.1309	62	99	114	YLYFHLQQQHIELIR					Mascot
2114.1545	2114.2854	0.1309	62	99	114	YLYFHLQQQHIELIR	53	99.953			Mascot
2161.0693	2161.21	0.1407	65	51	69	MESGIEPSVDLETLDERIK					Mascot
2177.0642	2177.1719	0.1077	49	51	69	MESGIEPSVDLETLDERIK		Oxidation (M)[1]			Mascot
2223.0598	2223.2134	0.1536	69	49	67	FRMESGIEPSVDLETLDERR					Mascot
2239.0547	2239.2361	0.1814	81	49	67	FRMESGIEPSVDLETLDERR		Oxidation (M)[3]			Mascot
2337.084	2337.2317	0.1477	63	117	137	ETEAALEFAQTQLAEQGEESR	28	84.548			Mascot
2337.084	2337.2317	0.1477	63	117	137	ETEAALEFAQTQLAEQGEESR					Mascot
2364.1465	2364.3118	0.1653	70	172	191	VWSEVNQAVLDYENRESTPK					Mascot
2364.1465	2364.3118	0.1653	70	172	191	VWSEVNQAVLDYENRESTPK					Mascot
2445.3096	2445.4861	0.1765	72	77	98	GQIQEALIALINSLHPELLDTNR	89	100			Mascot
2445.3096	2445.4861	0.1765	72	77	98	GQIQEALIALINSLHPELLDTNR					Mascot

2 Chromosome 20 open reading frame 11 [Homo sapiens] gil21594655 492 100 26802.7 4.92 11

Peptide Information											
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank Result Type			
993.5588	993.6671	0.1083	109	19	26 LNNLHVQR						Mascot
1427.7603	1427.8761	0.1158	81	32	43 LIMNYLVTEGFK						Mascot
1470.7952	1470.8982	0.103	70	198	209 LLLWAQNELDQK						Mascot
1598.89	1599.0055	0.1155	72	198	210 LLLWAQNELDQKK	17	0				Mascot
1598.89	1599.0055	0.1155	72	198	210 LLLWAQNELDQKK	108	100				Mascot
1821.8766	1821.9969	0.1203	66	172	186 VWSEVNQAVLDYENR						Mascot
1821.8766	1821.9969	0.1203	66	172	186 VWSEVNQAVLDYENR						Mascot

1956.0146	1956.1411	0.1265	65	32	48	LIMNYLVTEGFKEAAEK			Mascot
2078.03	2078.1665	0.1365	66	170	186	QKVVSEVNQAVLDYENR			Mascot
2078.03	2078.1665	0.1365	66	170	186	QKVVSEVNQAVLDYENR	80	100	Mascot
2114.1545	2114.2854	0.1309	62	99	114	YLYFHLQQQHLIELIR			Mascot
2114.1545	2114.2854	0.1309	62	99	114	YLYFHLQQQHLIELIR	53	99.953	Mascot
2337.084	2337.2317	0.1477	63	117	137	ETEAALEFAQTQLAEQG EESR	28	84.548	Mascot
2337.084	2337.2317	0.1477	63	117	137	ETEAALEFAQTQLAEQG EESR			Mascot
2364.1465	2364.3118	0.1653	70	172	191	VWSEVNQAVLDYENRES TPK			Mascot
2364.1465	2364.3118	0.1653	70	172	191	VWSEVNQAVLDYENRES TPK			Mascot
2445.3096	2445.4861	0.1765	72	77	98	GQIQEALIALINSLHPELLD TNR	89	100	Mascot
2445.3096	2445.4861	0.1765	72	77	98	GQIQEALIALINSLHPELLD TNR			Mascot

3 unnamed protein product [Homo sapiens] gi|194388890 113 100 17780 5.86 7

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
993.5588	993.6671	0.1083	109	19	26 LNNLHVQR				Mascot
1427.7603	1427.8761	0.1158	81	32	43 LIMNYLVTEGFK				Mascot
1919.8903	1920.014	0.1237	64	51	67 MESGIEPSVDLETLDER				Mascot
1919.8903	1920.014	0.1237	64	51	67 MESGIEPSVDLETLDER	41	99.313		Mascot
1956.0146	1956.1411	0.1265	65	32	48 LIMNYLVTEGFKEAAEK				Mascot
2161.0693	2161.21	0.1407	65	51	69 MESGIEPSVDLETLDERI K				Mascot
2177.0642	2177.1719	0.1077	49	51	69 MESGIEPSVDLETLDERI K		Oxidation (M)[1]		Mascot
2223.0598	2223.2134	0.1536	69	49	67 FRMESGIEPSVDLETLDER R				Mascot
2239.0547	2239.2361	0.1814	81	49	67 FRMESGIEPSVDLETLDER R		Oxidation (M)[3]		Mascot
2337.126	2337.2317	0.1057	45	135	155 EVVQGCVHCLRVGTVDG HMAL		Carbamidomethyl (C)[6,9]		Mascot
2337.126	2337.2317	0.1057	45	135	155 EVVQGCVHCLRVGTVDG HMAL		Carbamidomethyl (C)[6,9]		Mascot

4 spectrin repeat containing, nuclear envelope 1, isoform CRA_d [Homo sapiens] gi|119568115 44 0 372748 5.1 18

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
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957.6091	957.6151	0.006	6	3082	3089	LLLKEVSR		Mascot
1449.6387	1449.8259	0.1872	129	1665	1676	QCASTVQQQEDR	Carbamidomethyl (C)[2]	Mascot
1470.7375	1470.8982	0.1607	109	2415	2425	LWQKFLDDYSR		Mascot
1598.7744	1599.0055	0.2311	145	2175	2186	QWEELCHQLSLR	Carbamidomethyl (C)[6]	Mascot
1598.8424	1599.0055	0.1631	102	2436	2451	TAAFPSSSGVYTVAK		Mascot
1873.9034	1873.8937	-0.0097	-5	2810	2826	GYMKLLGECSSSIDSVK	Carbamidomethyl (C)[9]	Mascot
1919.9531	1920.014	0.0609	32	1074	1090	ETQFHTELMAQASAVLK	Oxidation (M)[9]	Mascot
1919.9531	1920.014	0.0609	32	1074	1090	ETQFHTELMAQASAVLK	Oxidation (M)[9]	Mascot
1955.9644	1956.1411	0.1767	90	1521	1537	ELGQTWANLDHMHVGQLK	Oxidation (M)[12]	Mascot
1977.9838	1978.0541	0.0703	36	2735	2753	GAVGLSDVMIPESPEAYVK	Oxidation (M)[9]	Mascot
2094.1382	2094.1687	0.0305	15	1863	1879	FSKLQSFLHQTFLEK		Mascot
2135.9993	2136.1802	0.1809	85	2641	2658	ELELEDSAALSDLHWDR		Mascot
2337.2131	2337.2317	0.0186	8	2169	2186	VELLQRQWEELCHQLSLR	Carbamidomethyl (C)[12]	Mascot
2337.2131	2337.2317	0.0186	8	2169	2186	VELLQRQWEELCHQLSLR	Carbamidomethyl (C)[12]	Mascot
2445.1399	2445.4861	0.3462	142	2111	2130	LSQSLPDHHEELHAEQM RCK	Carbamidomethyl (C)[19]	Mascot
2445.1399	2445.4861	0.3462	142	2111	2130	LSQSLPDHHEELHAEQM RCK	Carbamidomethyl (C)[19]	Mascot
2620.2744	2620.4973	0.2229	85	5	26	LQYLTSVYCTEKMSQQV AELGR	Carbamidomethyl (C)[9], Oxidation (M)[13]	Mascot
2620.3577	2620.4973	0.1396	53	1379	1401	QLTVDVNQSVLQISSQD VESKR		Mascot
2672.3276	2672.5518	0.2242	84	2730	2753	DFYLRGAVGLSDVMIPES PEAYVK	Oxidation (M)[14]	Mascot
2734.4258	2734.5615	0.1357	50	2151	2174	DTLSAYISADDISILNERV ELLQR		Mascot
2750.4321	2750.5693	0.1372	50	816	839	LFVATALLPEETETCLFN QEILAK	Carbamidomethyl (C)[15]	Mascot

5 spectrin repeat containing, nuclear envelope 1, isoform CRA_f [Homo sapiens] gi|119568117 42 0 385227.6 5.12 18

Peptide Information									
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank Result Type
957.6091	957.6151	0.006	6	3191	3198	LLLKEVSR			Mascot
1449.6387	1449.8259	0.1872	129	1774	1785	QCASTVQQQEDR	Carbamidomethyl (C)[2]		Mascot
1470.7375	1470.8982	0.1607	109	2524	2534	LWQKFLDDYSR			Mascot
1598.7744	1599.0055	0.2311	145	2284	2295	QWEELCHQLSLR	Carbamidomethyl (C)[6]		Mascot
1598.8424	1599.0055	0.1631	102	2545	2560	TAAFPSSSGVYTVAK			Mascot
1873.9034	1873.8937	-0.0097	-5	2919	2935	GYMKLLGECSSSIDSVK	Carbamidomethyl (C)[9]		Mascot
1919.9531	1920.014	0.0609	32	1183	1199	ETQFHTELMAQASAVLK	Oxidation (M)[9]		Mascot

1919.9531	1920.014	0.0609	32	1183	1199	ETQFHTELMAQASAVLK	Oxidation (M)[9]	Mascot
1955.9644	1956.1411	0.1767	90	1630	1646	ELGQTWANLDHDMVGQLK	Oxidation (M)[12]	Mascot
1977.9838	1978.0541	0.0703	36	2844	2862	GAVGLSDVMIPESPEAYVK	Oxidation (M)[9]	Mascot
2094.1382	2094.1687	0.0305	15	1972	1988	FSKLQSFLHQTFLEK		Mascot
2135.9993	2136.1802	0.1809	85	2750	2767	ELELEDSAALSDLHWDR		Mascot
2337.2131	2337.2317	0.0186	8	2278	2295	VELLQRQWEELCHQLSLR	Carbamidomethyl (C)[12]	Mascot
2337.2131	2337.2317	0.0186	8	2278	2295	VELLQRQWEELCHQLSLR	Carbamidomethyl (C)[12]	Mascot
2445.1399	2445.4861	0.3462	142	2220	2239	LSQSLPDHHEELHAEQM RCK	Carbamidomethyl (C)[19]	Mascot
2445.1399	2445.4861	0.3462	142	2220	2239	LSQSLPDHHEELHAEQM RCK	Carbamidomethyl (C)[19]	Mascot
2620.2744	2620.4973	0.2229	85	114	135	LQYLTSVYCTEKMSQQV AELGR	Carbamidomethyl (C)[9], Oxidation (M)[13]	Mascot
2620.3577	2620.4973	0.1396	53	1488	1510	QLTVDFVNQSVLQISSQD VESKR		Mascot
2672.3276	2672.5518	0.2242	84	2839	2862	DFYLRGAVGLSDVMIPES PEAYVK	Oxidation (M)[14]	Mascot
2734.4258	2734.5615	0.1357	50	2260	2283	DTLSAYISADDISILNERV ELLQR		Mascot
2750.4321	2750.5693	0.1372	50	925	948	LFVATALLPEETETCLFN QEILAK	Carbamidomethyl (C)[15]	Mascot

6 DNAH10 protein [Homo sapiens] gi|229597159 42 0 114096.1 6.5 10

Protein Group
DNAH10 protein [Homo sapiens]

gi|229597161 114096.1 6.5

Peptide Information	Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result Type
	993.5978	993.6671	0.0693	70	742	749	KIYEVLTK				Mascot
	1470.7661	1470.8982	0.1321	90	381	393	SAEAAFDMLLKFK				Mascot
	1853.8278	1853.9741	0.1463	79	792	805	MCFHCVRNCVEITK		Carbamidomethyl (C)[2,5,9]		Mascot
	1873.9663	1873.8937	-0.0726	-39	205	219	MIPLMERIAWEIAER		Oxidation (M)[1]		Mascot
	1955.9644	1956.1411	0.1767	90	16	31	DEFLMNVQKFASNIQR		Oxidation (M)[5]		Mascot
	2177.0002	2177.1719	0.1717	79	954	970	EELYNLHEEMEVLNRCV		Carbamidomethyl (C)[16]		Mascot
	2223.1189	2223.2134	0.0945	43	498	515	YEQWMEMVTEQVLPALMK K				Mascot
	2239.1138	2239.2361	0.1223	55	498	515	YEQWMEMVTEQVLPALMK K		Oxidation (M)[5]		Mascot
	2445.2153	2445.4861	0.2708	111	586	606	MLDHYHMLIGTLNDAESV LLK		Oxidation (M)[1,7]		Mascot
	2445.2153	2445.4861	0.2708	111	586	606	MLDHYHMLIGTLNDAESV LLK		Oxidation (M)[1,7]		Mascot

2467.2585	2467.3606	0.1021	41	168	190	NITHGSGFHVVLDTIPAM MSALR			Mascot
2689.3137	2689.5137	0.2	74	947	968	LLNESAKEEELYNLHEEME VLNR	Oxidation (M)[17]		Mascot

7 IFT74 protein [Homo sapiens] gi|211827211 40 0 57874.5 5.83 7

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result Type
1598.818	1599.0055	0.1875	0.1875	117	1	15 MASNHKSSAARPVSR				Mascot
1598.818	1599.0055	0.1875	0.1875	117	1	15 MASNHKSSAARPVSR				Mascot
1948.9354	1949.0439	0.1085	0.1085	56	456	471 MELLESKMTEEQHSLK		Oxidation (M)[1]		Mascot
2061.1675	2061.1501	-0.0174	-0.0174	-8	16	36 GGVGLTGRPPSGIRPLS GNIR				Mascot
2094.0979	2094.1687	0.0708	0.0708	34	272	288 QEAVLLHEKLYELESHR				Mascot
2161.0806	2161.21	0.1294	0.1294	60	240	257 LLQELDTLQQQLDSQNM K		Oxidation (M)[17]		Mascot
2223.0386	2223.2134	0.1748	0.1748	79	118	135 GIEMYNQENSVYLSYEK R				Mascot
2239.0334	2239.2361	0.2027	0.2027	91	118	135 GIEMYNQENSVYLSYEK R		Oxidation (M)[4]		Mascot
2718.3616	2718.5452	0.1836	0.1836	68	235	257 TTNEKLLQELDTLQQQLD SQNMK				Mascot
2718.3616	2718.5452	0.1836	0.1836	68	235	257 TTNEKLLQELDTLQQQLD SQNMK				Mascot
2734.3564	2734.5615	0.2051	0.2051	75	235	257 TTNEKLLQELDTLQQQLD SQNMK		Oxidation (M)[22]		Mascot

8 IFT74 protein [Homo sapiens] gi|78070721 38 0 42362.5 5.46 6

Protein Group

intraflagellar transport protein 74 homolog isoform b [Homo sapiens]	gi 151101151	42362.5	5.4600 000381 4697
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Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result Type
1598.818	1599.0055	0.1875	0.1875	117	1	15 MASNHKSSAARPVSR				Mascot
1598.818	1599.0055	0.1875	0.1875	117	1	15 MASNHKSSAARPVSR				Mascot
2061.1675	2061.1501	-0.0174	-0.0174	-8	16	36 GGVGLTGRPPSGIRPLS GNIR				Mascot
2094.0979	2094.1687	0.0708	0.0708	34	272	288 QEAVLLHEKLYELESHR				Mascot
2161.0806	2161.21	0.1294	0.1294	60	240	257 LLQELDTLQQQLDSQNM K		Oxidation (M)[17]		Mascot
2223.0386	2223.2134	0.1748	0.1748	79	118	135 GIEMYNQENSVYLSYEK R				Mascot
2239.0334	2239.2361	0.2027	0.2027	91	118	135 GIEMYNQENSVYLSYEK R		Oxidation (M)[4]		Mascot

2718.3616	2718.5452	0.1836	68	235	257	TTNEKLLQELDTLQQQLD SQNMK		Mascot
2718.3616	2718.5452	0.1836	68	235	257	TTNEKLLQELDTLQQQLD SQNMK		Mascot
2734.3564	2734.5615	0.2051	75	235	257	TTNEKLLQELDTLQQQLD SQNMK	Oxidation (M)[22]	Mascot

9 KIAA0796 protein [Homo sapiens] gi|20521662 38 0 217930.8 5.32 13

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result Type
957.6091	957.6151	0.006	6	1723	1730	LLLKEVSR				Mascot
1449.6387	1449.8259	0.1872	129	329	340	QCASTVQQQEDR		Carbamidomethyl (C)[2]		Mascot
1470.7375	1470.8982	0.1607	109	1079	1089	LWQKFDDYSR				Mascot
1598.7744	1599.0055	0.2311	145	839	850	QWEELCHQLSLR		Carbamidomethyl (C)[6]		Mascot
1598.8424	1599.0055	0.1631	102	1100	1115	TAAFPSSSGVIYTVAK				Mascot
1873.9034	1873.8937	-0.0097	-5	1451	1467	GYMKLLGECSSSIDSVK		Carbamidomethyl (C)[9]		Mascot
1955.9644	1956.1411	0.1767	90	185	201	ELGQTWANLDHMVGQLK		Oxidation (M)[12]		Mascot
2094.1382	2094.1687	0.0305	15	527	543	FSKLQSFLHQHTFLEK				Mascot
2135.9993	2136.1802	0.1809	85	1305	1322	ELELEDASAALSDLHWHD R				Mascot
2337.2131	2337.2317	0.0186	8	833	850	VELLQRQWEELCHQLSL R		Carbamidomethyl (C)[12]		Mascot
2337.2131	2337.2317	0.0186	8	833	850	VELLQRQWEELCHQLSL R		Carbamidomethyl (C)[12]		Mascot
2445.1399	2445.4861	0.3462	142	775	794	LSQSLPDHHEELHAEQM RCK		Carbamidomethyl (C)[19]		Mascot
2445.1399	2445.4861	0.3462	142	775	794	LSQSLPDHHEELHAEQM RCK		Carbamidomethyl (C)[19]		Mascot
2620.3577	2620.4973	0.1396	53	43	65	QLTVDFVNQSVLQISSQD VESKR				Mascot
2620.3577	2620.4973	0.1396	53	43	65	QLTVDFVNQSVLQISSQD VESKR				Mascot
2734.4258	2734.5615	0.1357	50	815	838	DTLSAYISADDISILNERV ELLQR				Mascot

10 hCG1643112, isoform CRA_b [Homo sapiens] gi|119585114 38 0 58339.8 8.52 6

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result Type
1449.8074	1449.8259	0.0185	13	286	297	NLVRHQFLHTGK				Mascot
1598.9125	1599.0055	0.093	58	196	208	TFRQTSQQLIVHLR				Mascot
1598.9125	1599.0055	0.093	58	196	208	TFRQTSQQLIVHLR				Mascot
1821.7894	1821.9969	0.2075	114	181	195	IHTGEKPYECNECGK		Carbamidomethyl (C)[10,13]		Mascot
1821.7894	1821.9969	0.2075	114	433	447	IHTGEKPYECNECGK		Carbamidomethyl (C)[10,13]		Mascot

1977.8905	1978.0541	0.1636	83	180	195	RIHTGEKPYECNECGK	Carbamidomethyl (C)[11,14]	Mascot
2222.9858	2223.2134	0.2276	102	162	179	CDECGKAFYWSSHLIGH R	Carbamidomethyl (C)[1,4]	Mascot
3386.5352	3386.8064	0.2712	80	433	460	IHTGEKPYECNECGKVFS YNSSLMVHQQR	Carbamidomethyl (C)[10,13], Oxidation (M)[24]	Mascot

11 PREDICTED: hypothetical protein LOC100505970 [Homo sapiens] gi|310123740 38 0 14232.3 11.15 4

Protein Group

PREDICTED: hypothetical protein LOC100505970 [Homo sapiens]

unnamed protein product [Homo sapiens]

gi|341914099 14232.3 11.149
999618
5303

gi|34526672 14232.3 11.149
999618
5303

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result	Type
1449.7631	1449.8259	0.0628	43	113	124	MVQFGLTPRTQR		Oxidation (M)[1]			Mascot
2078.0376	2078.1665	0.1289	62	88	106	MFLTAQTPWNVSLQPGS GK		Oxidation (M)[1]			Mascot
2078.0376	2078.1665	0.1289	62	88	106	MFLTAQTPWNVSLQPGS GK		Oxidation (M)[1]			Mascot
2161.0134	2161.21	0.1966	91	33	50	DVPMMRQLGPCSQQNMI R		Carbamidomethyl (C)[11]			Mascot
2177.0083	2177.1719	0.1636	75	33	50	DVPMMRQLGPCSQQNMI R		Carbamidomethyl (C)[11], Oxidation (M)[4]			Mascot
2718.3459	2718.5452	0.1993	73	39	61	QLGPCSQQNMRMLQL GLCPAR		Carbamidomethyl (C)[5,20]			Mascot
2718.3459	2718.5452	0.1993	73	39	61	QLGPCSQQNMRMLQL GLCPAR		Carbamidomethyl (C)[5,20]			Mascot
2734.3408	2734.5615	0.2207	81	39	61	QLGPCSQQNMRMLQL GLCPAR		Carbamidomethyl (C)[5,20], Oxidation (M)[10]			Mascot
2750.3357	2750.5693	0.2336	85	39	61	QLGPCSQQNMRMLQL GLCPAR		Carbamidomethyl (C)[5,20], Oxidation (M)[10,13]			Mascot

12 unnamed protein product [Homo sapiens] gi|40038701 38 0 13205.4 8.4 4

Protein Group

hCG2021286 [Homo sapiens]

gi|119598724 13205.4 8.3999
996185
3027

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result	Type
1449.7269	1449.8259	0.099	68	1	11	MYLYCQIAFLK		Carbamidomethyl (C)[5]			Mascot
1821.8549	1821.9969	0.142	78	93	109	TGSCSVAQAGVQWRDL S		Carbamidomethyl (C)[4]			Mascot
1821.8549	1821.9969	0.142	78	93	109	TGSCSVAQAGVQWRDL		Carbamidomethyl (C)[4]			Mascot

							S				
2445.1433	2445.4861	0.3428	140	54	71	REELNCVCLLCLEIYMWK		Carbamidomethyl (C)[6,8,11], Oxidation (M)[16]		Mascot	
2445.1433	2445.4861	0.3428	140	55	72	EELNCVCLLCLEIYMWKR		Carbamidomethyl (C)[5,7,10], Oxidation (M)[15]		Mascot	
13	immunoglobulin gamma heavy chain [Homo sapiens]						gi 1419579	37	0	14149	7.93
										4	
Peptide Information											
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result	Type
1956.0582	1956.1411	0.0829	42	1	19	QVQLVQAGAEVMKPGSS VK				Mascot	
2222.9336	2223.2134	0.2798	126	88	105	SEDTAVYYCAVYYFDSN R		Carbamidomethyl (C)[9]		Mascot	
2430.2842	2429.948	-0.3362	-138	1	23	QVQLVQAGAEVMKPGSS VKVSCK		Carbamidomethyl (C)[22]		Mascot	
2672.3606	2672.5518	0.1912	72	106	130	YDYWGQQGTLVIVSSAST KGPSVFPL				Mascot	
14	RecName: Full=Putative zinc finger protein 852						gi 298286894	37	0	59553.6	8.46
										6	
Peptide Information											
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result	Type
1449.8074	1449.8259	0.0185	13	251	262	NLVRHQFLHTGK				Mascot	
1598.9125	1599.0055	0.093	58	161	173	TFRQTSQLIVHLR				Mascot	
1598.9125	1599.0055	0.093	58	161	173	TFRQTSQLIVHLR				Mascot	
1821.7894	1821.9969	0.2075	114	146	160	IHTGEKPYECNECGK		Carbamidomethyl (C)[10,13]		Mascot	
1821.7894	1821.9969	0.2075	114	398	412	IHTGEKPYECNECGK		Carbamidomethyl (C)[10,13]		Mascot	
1977.8905	1978.0541	0.1636	83	145	160	RIHTGEKPYECNECGK		Carbamidomethyl (C)[11,14]		Mascot	
2222.9858	2223.2134	0.2276	102	127	144	CDEC GKAFYWSSHLIGH R		Carbamidomethyl (C)[1,4]		Mascot	
3386.5352	3386.8064	0.2712	80	398	425	IHTGEKPYECNECGKVFS YNSSLMVHQR		Carbamidomethyl (C)[10,13], Oxidation (M)[24]		Mascot	
15	unnamed protein product [Homo sapiens]						gi 34531580	37	0	107163.5	6.32
										9	
Peptide Information											
Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Seq.	Sequence	Ion Score	C. I. % Modification	Rank	Result	Type
993.5978	993.6671	0.0693	70	685	692	KIYEVLTK				Mascot	
1853.8278	1853.9741	0.1463	79	735	748	MCFHCVRNCVEITK		Carbamidomethyl (C)[2,5,9]		Mascot	
1873.9663	1873.8937	-0.0726	-39	205	219	MIPLMERIAWEIAER		Oxidation (M)[1]		Mascot	
1955.9644	1956.1411	0.1767	90	16	31	DEFLMNVQKFASNIQR		Oxidation (M)[5]		Mascot	
2177.0002	2177.1719	0.1717	79	897	913	EELYNLHEEMEVLNRCV		Carbamidomethyl (C)[16]		Mascot	

2223.1189	2223.2134	0.0945	43	441	458	YEQWMEVTEQVLPALMK K			Mascot
2239.1138	2239.2361	0.1223	55	441	458	YEQWMEVTEQVLPALMK K	Oxidation (M)[5]		Mascot
2445.2153	2445.4861	0.2708	111	529	549	MLDHYHMLIGTLNDAESV LLK	Oxidation (M)[1,7]		Mascot
2445.2153	2445.4861	0.2708	111	529	549	MLDHYHMLIGTLNDAESV LLK	Oxidation (M)[1,7]		Mascot
2467.2585	2467.3606	0.1021	41	168	190	NITHGSGFHVVLDTIPAM MSALR			Mascot
2689.3137	2689.5137	0.2	74	890	911	LLNESAKEEYLNHEEMEV LNR	Oxidation (M)[17]		Mascot

16 unnamed protein product [Homo sapiens] gi|40033395 37 0 56339.4 6.78 7

Protein Group

unnamed protein product [Homo sapiens]

gi|10432996 56339.4 6.7800
002098
0835

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
957.4974	957.6151	0.1177	123	241	247 YMNFRVK				Mascot
1449.7518	1449.8259	0.0741	51	119	131 QIKDGVTMAPAFR		Oxidation (M)[8]		Mascot
1470.7474	1470.8982	0.1508	103	19	30 NEEIQSFIYSLK				Mascot
1492.6737	1492.8605	0.1868	125	283	295 VDDLSVEWDAMGR				Mascot
1620.7687	1620.95	0.1813	112	283	296 VDDLSVEWDAMGRK				Mascot
2077.9795	2078.1665	0.187	90	136	153 AKVSDNMMSHLMDFAQE R				Mascot
2077.9795	2078.1665	0.187	90	136	153 AKVSDNMMSHLMDFAQE R				Mascot
2093.9744	2094.1687	0.1943	93	136	153 AKVSDNMMSHLMDFAQE R		Oxidation (M)[7]		Mascot
2672.2847	2672.5518	0.2671	100	248	271 ACNKAVAGEFSEPVTLET PAFMFR		Carbamidomethyl (C)[2]		Mascot

17 PREDICTED: LOW QUALITY PROTEIN: zinc finger protein 167 [Homo sapiens] gi|239742001 36 0 62253.6 8.59 6

Protein Group

PREDICTED: zinc finger protein 167 [Homo sapiens]

gi|341914472 62253.6 8.5900
001525
8789

unnamed protein product [Homo sapiens]

gi|194384678 62263.6 8.5900
001525
8789

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
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	1449.8074	1449.8259	0.0185	13	285	296	NLVRHQFLHTGK		Mascot			
	1598.9125	1599.0055	0.093	58	195	207	TFRQTSQLIVHLR		Mascot			
	1598.9125	1599.0055	0.093	58	195	207	TFRQTSQLIVHLR		Mascot			
	1821.7894	1821.9969	0.2075	114	180	194	IHTGEKPYECNECGK	Carbamidomethyl (C)[10,13]	Mascot			
	1821.7894	1821.9969	0.2075	114	432	446	IHTGEKPYECNECGK	Carbamidomethyl (C)[10,13]	Mascot			
	1977.8905	1978.0541	0.1636	83	179	194	RIHTGEKPYECNECGK	Carbamidomethyl (C)[11,14]	Mascot			
	2222.9858	2223.2134	0.2276	102	161	178	CDECYGAFYWSSHLIGH R	Carbamidomethyl (C)[1,4]	Mascot			
	3386.5352	3386.8064	0.2712	80	432	459	IHTGEKPYECNECGKVFS YNSSLMVHQR	Carbamidomethyl (C)[10,13], Oxidation (M)[24]	Mascot			
18	transcription activator [Homo sapiens]						gi 292498	36	0	89010.8	6.98	8

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
957.5727	957.6151	0.0424	44	632	639 IVERAEIK				Mascot
1470.7773	1470.8982	0.1209	82	740	751 LGMVEWIEPPKR		Oxidation (M)[3]		Mascot
1821.8348	1821.9969	0.1621	89	149	163 TEQEEDDELLSESRK				Mascot
1821.8348	1821.9969	0.1621	89	149	163 TEQEEDDELLSESRK				Mascot
2078.0134	2078.1665	0.1531	74	681	698 ESELTDEDITTLERGEK				Mascot
2078.0134	2078.1665	0.1531	74	681	698 ESELTDEDITTLERGEK				Mascot
2101.0317	2101.1072	0.0755	36	663	680 EEMLQMIRHGATHVFAS K		Oxidation (M)[3]		Mascot
2467.0171	2467.3606	0.3435	139	276	295 DEMMPGEWDVCVTSYE MVIK		Carbamidomethyl (C)[11], Oxidation (M)[3,4,17]		Mascot
2689.374	2689.5137	0.1397	52	230	252 NIPGPHMVLVPKSTLHN WMNEFK				Mascot
2718.3452	2718.5452	0.2	74	505	525 VLIFSQMTRLLDILEDYCM WR		Carbamidomethyl (C)[18], Oxidation (M)[7]		Mascot
2718.3452	2718.5452	0.2	74	505	525 VLIFSQMTRLLDILEDYCM WR		Carbamidomethyl (C)[18], Oxidation (M)[7]		Mascot
2734.3401	2734.5615	0.2214	81	505	525 VLIFSQMTRLLDILEDYCM WR		Carbamidomethyl (C)[18], Oxidation (M)[7,19]		Mascot

19	TPA_exp: spectrin-like protein of the nuclear envelope and Golgi [Homo sapiens]						gi 34849883	36	0	972409.3	5.4	26
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Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
957.6091	957.6151	0.006	6	8254	8261 LLLKEVSR				Mascot
1449.6387	1449.8259	0.1872	129	6860	6871 QCASTVQQQEDR		Carbamidomethyl (C)[2]		Mascot
1470.7434	1470.8982	0.1548	105	4129	4142 SDAESTAVHLEALK				Mascot

1492.7716	1492.8605	0.0889	60	738	750	LSEPLEVSFMNVK		Mascot
1598.8384	1599.0055	0.1671	105	4129	4143	SDAESTAVHLEALKK		Mascot
1598.8424	1599.0055	0.1631	102	7631	7646	TAAFPSSSGVIYTVAK		Mascot
1620.8666	1620.95	0.0834	51	737	750	KLSEPLEVSFMNVK		Mascot
1821.9204	1821.9969	0.0765	42	3571	3584	LMEQKFQQVDEWLK		Mascot
1821.9204	1821.9969	0.0765	42	3571	3584	LMEQKFQQVDEWLK		Mascot
1837.9153	1837.9769	0.0616	34	3571	3584	LMEQKFQQVDEWLK	Oxidation (M)[2]	Mascot
1873.9034	1873.8937	-0.0097	-5	7982	7998	GYMKLLGECSIDSVK	Carbamidomethyl (C)[9]	Mascot
1919.9531	1920.014	0.0609	32	6269	6285	ETQFHTELMAQASAVLK	Oxidation (M)[9]	Mascot
1919.9531	1920.014	0.0609	32	6269	6285	ETQFHTELMAQASAVLK	Oxidation (M)[9]	Mascot
1956.0371	1956.1411	0.104	53	2592	2608	NEVHKVEMFLGELLAAR		Mascot
1977.9916	1978.0541	0.0625	32	4896	4912	ILEDADVDEWTGFNNKVK		Mascot
2061.0168	2061.1501	0.1333	65	4914	4932	ATEMIDQLQDKLPGSSAEK		Mascot
2094.1382	2094.1687	0.0305	15	7058	7074	FSKLQSFLLHQHTFLEK		Mascot
2100.9482	2101.1072	0.159	76	2447	2463	HFSESMQEFAQWFLGAK		Mascot
2135.9993	2136.1802	0.1809	85	7836	7853	ELELEDSAALSDLHWHD R		Mascot
2161.1863	2161.21	0.0237	11	841	859	INEIITVLEREAQSSALFK		Mascot
2177.0767	2177.1719	0.0952	44	1608	1626	EHAQCLEGTLGHLSQQ QK	Carbamidomethyl (C)[5]	Mascot
2223.1404	2223.2134	0.073	33	2807	2825	FVLLDHLQSQILSEAEDHT R		Mascot
2239.0701	2239.2361	0.166	74	4003	4020	SELWIYLQDADQQLQNM K	Oxidation (M)[17]	Mascot
2337.2131	2337.2317	0.0186	8	7364	7381	VELLQRQWEELCHQLSL R	Carbamidomethyl (C)[12]	Mascot
2337.2131	2337.2317	0.0186	8	7364	7381	VELLQRQWEELCHQLSL R	Carbamidomethyl (C)[12]	Mascot
2445.2441	2445.4861	0.242	99	4023	4043	HSELELNIAQNMVSQVKDFVK	Oxidation (M)[12]	Mascot
2445.2441	2445.4861	0.242	99	4023	4043	HSELELNIAQNMVSQVKDFVK	Oxidation (M)[12]	Mascot
2620.293	2620.4973	0.2043	78	2338	2359	DFTAQSTQVEKFINDITTWFTK		Mascot
2620.3577	2620.4973	0.1396	53	6574	6596	QLTVDVFVNQSVLQISSQDVESKR		Mascot
2718.4312	2718.5452	0.114	42	878	902	MTLCLVLGSMISMLSTAKI TIAGYK	Carbamidomethyl (C)[4], Oxidation (M)[1]	Mascot
2718.4312	2718.5452	0.114	42	878	902	MTLCLVLGSMISMLSTAKI TIAGYK	Carbamidomethyl (C)[4], Oxidation (M)[1]	Mascot
2734.426	2734.5615	0.1355	50	878	902	MTLCLVLGSMISMLSTAKI TIAGYK	Carbamidomethyl (C)[4], Oxidation (M)[1,10]	Mascot
2750.4321	2750.5693	0.1372	50	6011	6034	LFVATALLPEETETCLFN QEILAK	Carbamidomethyl (C)[15]	Mascot

20 malignant T cell amplified sequence 1, isoform CRA_b
[Homo sapiens]

gi|119632280

36 0 15684.2 8.98 4

Peptide Information

Calc. Mass	Obsrv. Mass	± da	± ppm	Start Seq.	End Sequence Seq.	Ion Score	C. I. % Modification	Rank	Result Type
1854.0272	1853.9741	-0.0531	-29	45	60 YPFILPHQQVDKGAIK				Mascot
1919.9718	1920.014	0.0422	22	61	79 FVLSGANIMCPGLTSPGA K		Carbamidomethyl (C)[10]		Mascot
1919.9718	1920.014	0.0422	22	61	79 FVLSGANIMCPGLTSPGA K		Carbamidomethyl (C)[10]		Mascot
2061.9766	2061.6851	-0.2915	-141	97	114 QHALCVGVVMKMSAEDIE K		Carbamidomethyl (C)[5], Oxidation (M)[9]		Mascot
2077.9714	2078.1665	0.1951	94	97	114 QHALCVGVVMKMSAEDIE K		Carbamidomethyl (C)[5], Oxidation (M)[9,11]		Mascot
2077.9714	2078.1665	0.1951	94	97	114 QHALCVGVVMKMSAEDIE K		Carbamidomethyl (C)[5], Oxidation (M)[9,11]		Mascot
2467.2551	2467.3606	0.1055	43	115	135 VNKGIGIENIHYLNDGLW HMK		Oxidation (M)[20]		Mascot