A Tandem Affinity Tag for Two-step Purification under Fully Denaturing Conditions

APPLICATION IN UBIQUITIN PROFILING AND PROTEIN COMPLEX IDENTIFICATION COMBINED WITH IN VIVO CROSS-LINKING

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Tandem affinity strategies reach exceptional protein purification grades and have considerably improved the outcome of mass-spectrometry-based proteomic experiments. However, current tandem affinity tags are incompatible with two-step purification under fully denaturing conditions. Such stringent purification conditions are desirable for mass-spectrometric analyses of protein modifications as they result in maximal preservation of posttranslational modifications. Here we describe the histidine-biotin (HB) tag, a new tandem affinity tag for two-step purification under denaturing conditions. The HB tag consists of a hexahistidine tag and a bacterially derived in vivo biotinylation signal peptide that induces efficient biotin attachment to the HB tag in yeast and mammalian cells. HB-tagged proteins can be sequentially purified under fully denaturing conditions, such as 8 M urea, by Ni²⁺ chelate chromatography and binding to streptavidin resins. The stringent separation conditions compatible with the HB tag prevent loss of protein modifications, and the high purification grade achieved by the tandem affinity strategy facilitates mass-spectrometric analysis of posttranslational modifications. Ubiquitination is a particularly sensitive protein modification that is rapidly lost during purification under native conditions due to ubiquitin hydrolase activity. The HB tag is ideal to study ubiquitination because the denaturing conditions inhibit hydrolase activity, and the tandem affinity strategy greatly reduces nonspecific background. We tested the HB tag in proteome-wide ubiquitin profiling experiments in yeast and identified a number of known ubiquitinated proteins as well as so far unidentified candidate ubiquitination targets. In addition, the stringent purification conditions compatible with the HB tag allow effective mass-spectrometric identification of in vivo cross-linked protein complexes, thereby expanding proteomic analyses to the description of weakly or transiently associated protein complexes. Molecular & Cellular Proteomics 5:737–748, 2006.

Mass spectrometric analysis of proteins has tremendously contributed to our understanding of biological systems. Mapping of covalent protein modifications by mass spectrometric approaches has made it possible to identify and rapidly evaluate the biological significance of modifications. In addition, identification of protein complexes by mass spectrometry has allowed investigators to connect cellular pathways and to describe the dynamics of protein complexes (1, 2). These approaches typically require a high degree of purification of proteins or protein complexes. Importantly to get a genuine picture of the in vivo situation it is essential to avoid any changes in protein modification or protein complex composition that might occur during the purification procedure.

Two-step purification strategies have been proven to be very effective in reducing nonspecific background, which is particularly important for the analyses of complex protein samples (3). The first widely and successfully used tandem affinity tag was the TAP1 tag, which consists of the immunoglobulin-interacting domain of Protein A and a calmodulin-binding peptide (CBP) and allows sequential purification based on Protein A/IgG-agarose and CBP/calmodulin-bead affinities (4). Other tandem affinity tags include a modified version of the TAP tag in which the CBP part is replaced by the S-tag (5) and combinations of multihistidine tags with FLAG (6, 7) or Myc tags (8).

The available tandem affinity purification strategies require native conditions in at least one of the purification steps and are therefore susceptible to loss of posttranslational modifications during cell lysis and purification because modifying as well as demodifying enzymes remain active under these con-

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1 The abbreviations used are: TAP, tandem affinity purification; CBP, calmodulin-binding peptide; HB, histidine-biotin; HBH, histidine-biotin-histidine; HBT, histidine-biotin-TEV; 1-D, one-dimensional; SCX, strong cation exchange; GO, Gene Ontology; TEV, tobacco etch virus; HRP, horseradish peroxidase; SUMO, small ubiquitin-like modifier.
ditions. To overcome these limitations we developed a tandem affinity tag, the HB tag, that allows two-step purification under fully denaturing conditions.

**EXPERIMENTAL PROCEDURES**

**Plasmids, Growth Media, and Yeast Strains**—Yeast strains used in this study are all isogenic to 15dau8b, bar1Δ ura3Δ His3, a derivative of BF264-15D (9). Strains were grown in standard culture medium, and standard yeast genetic methods were used (10). Carboxyl-terminal tagging of Skp1 and Met4 was performed by a PCR-based method as described previously (11). For this purpose the HBH tag was inserted into the pFA6a-kanMX6 plasmid to generate a PCR template for the PCR-based HBH tagging (12). Similar PCR tagging templates were constructed with other HB tag modules described in Fig. 1A. A BamHI fragment containing HBT-ubiquitin was inserted into the yeast expression vector YEpURA-CUP, which contains the inducible CUP1 promoter (region —463 to +1 of the CUP1 gene) and the termination sequence from the CYC1 gene (region +328 to +552) in the YEplac195 vector backbone (13). To express HBT-ubiquitin in mammalian cells a BamHI fragment containing the HBT tag fused to ubiquitin was inserted into pcDNA3.1 (Invitrogen).

**Two-step Purification of HB-tagged Proteins**—Cells expressing either carboxyl-terminal HBH-tagged Met4 (strain PY1228) or untagged Met4 (PY236) were grown in 300 ml of yeast peptone, 2% dextrose) to 0.8. Expression of untagged or HBT-ubiquitin was measured for 3 h by addition of CuSO4 (100 μM final concentration).

**LC/MS Analysis**—For one-dimensional (1-D) LC MS/MS analysis, the tryptic digest was directly injected onto the column, whereas the Lys-C/trypsin digests were desalted first with a C18 Ziptip (Millipore) before analysis. 1-D LC MS/MS was carried out by nanoflow reverse phase LC (Ultimate, LC Packings-Dionex) coupled to a quadrupole-orthogonal-time-of-flight tandem mass spectrometer (QSTAR XL, Applied Biosystems/MD Sciex). Reverse phase LC was performed using a PepMap column (75-μm inner diameter × 150 mm long, LC Packings-Dionex), and the peptides were eluted using a linear gradient of 0–35% solvent B in 100 min at a flow of 200 nl/min. Solvent A contained 98% H2O, 2%acetonitrile, 0.1% formic acid; solvent B was composed of 98% acetonitrile, 2% H2O, 0.1% formic acid. The QSTAR mass spectrometer was operated in an information-dependent mode in which each full MS scan was followed by three MS/MS scans where the three most abundant peptide molecular ions were dynamically selected for CID, thus generating tandem mass spectra. In general, the ions selected for CID were the most abundant in the MS spectrum except that singly charged ions were excluded, and dynamic exclusion was used to prevent repetitive selection of the same ions within a preset time. Collision energies were programmed to be adjusted automatically according to the charge state and mass value of the precursor ions. To increase the number of the MS/MS spectra acquired from any given sample and improve the dynamic range of mass spectrometric
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analysis, multiple LC MS/MS runs were performed on the same sample with exclusion lists generated from the previous LC MS/MS runs using the Mascot script within the Analyst program.

For the two-dimensional LC MS/MS, the digests were first separated by strong cation exchange (SCX) chromatography, which was performed using an AKTA system (Amersham Biosciences). Solvent A (5 mM KH2PO4, 30% acetonitrile, pH 3 adjusted with formic acid) and solvent B (solvent A with 350 mM KCl) were used to develop a salt gradient. The digests were separated by SCX chromatography using a 2.1 × 10-mm polysulfoethyl A Guard column (Poly LC, Columbia, MD) at a flow rate of 150 μl/min, and the separation profile was monitored by UV absorbance at 215, 254, and 280 nm, respectively. A typical separation used 0% B from 0 to 10 min to allow for sample loading and removal of non-peptide species followed by a gradient of 0–100% B from 25 to 30 min. Fractions were manually collected based on UV absorbance. All the SCX fractions were desalted offline using a C18 ZipTip (Millipore) prior to LC MS/MS.

The monoisotopic masses (m/z) of both parent ions and their corresponding fragment ions, parent ion charge states (z), and ion intensities from the MS/MS spectra acquired were automatically extracted using the script in the Analyst software and directly submitted for automated database searching for protein identification using two different search engines, Protein Prospector (University of California San Francisco) and Mascot (Matrix Science), to improve confidence levels of the protein identifications in the large datasets. The LC-Batch tag program within the developmental version of Protein Prospector was used for database searching. The mass accuracy for parent ions and fragment ions were set as ±100 and 300 ppm, respectively. An in-house Mascot program was also used for the database searching, and the mass accuracy for parent ions were set as ±100 ppm, and 0.3 Da was the fragment ion mass tolerance. The Swiss-Prot public database was queried to identify the purified proteins. In addition, the search Compare program within the developmental version of Protein Prospector (14) was used to make a list of proteins that differed between samples. Only the peptides identified with peptide score >25 in both Protein Prospector and Mascot search results were included for protein identification. Selected proteins were further validated by manual inspection of the MS/MS spectra. All the MS/MS spectra for the identified ubiquitinated peptides shown in Fig. 6 were manually examined.

Data Processing—To search the existing databases of protein annotations, Gene Ontology, and protein interactions, a Web interface (contact14.ics.uci.edu/swissstogene.html) was developed to convert the Swiss-Prot accession numbers into gene names and yeast ORFs using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB). This interface is built with Perl Common Gateway Interface (CGI) scripts. Furthermore a suite of Perl programs was used to process the output of our experiments, query the GOnet database (contact5.ics.uci.edu/gonet/sgidindex.html) (15), and format the output from GOnet. GOnet is a database of yeast protein interactions and Gene Ontology that allows users to rapidly cluster genes according to interaction and GO hierarchy. With the information retrieved from GOnet, we were able to rapidly classify the proteins according to their molecular functions, biological processes, and cellular components.

RESULTS

The His6–Bio Tandem Affinity Tag—There are only two affinity purification strategies we are aware of that tolerate fully denaturing conditions such as 8 M urea or 6 M guanidinium chloride, namely binding of a hexahistidine sequence to Ni2+–chelating resins and the interaction between biotin and the Streptomyces avidinii protein streptavidin. We therefore thought to combine these two purification steps into a tandem affinity tag to develop a two-step purification strategy that is compatible with completely denaturing conditions. To this end we combined a RGSH6 tag with a bacterially derived polypeptide that serves as a biotinylation signal in vivo (16) (Fig. 1). We refer to this tandem affinity tag as the HB tag. The nine-amino acid-long RGSH6 peptide was chosen because it combines affinity to Ni2+–chelate resins and immunodetection with commercially available antibodies with low cross-reactivity toward proteins present in eukaryotic cells. A specific lysine residue in the biotinylation signal serves as an efficient acceptor for biotin in vivo. Biotinylation is catalyzed by endogenous biotin ligases, which are present in all prokaryotic and eukaryotic cells. We tested several HB-tagged proteins in the yeast Saccharomyces cerevisiae as well as in human cell lines and found that the HB tag was quantitatively biotinylated in vivo (see below). In addition, no effect on cell morphology or cell viability was observed when eight different essential yeast genes were replaced by HB-tagged alleles indicating that the HB tag generally does not interfere with protein function (data not shown).

HB-tagged proteins can be sequentially purified by Ni2+–chelate chromatography and binding to streptavidin resins. The extraordinarily high affinity between biotin and streptavi-
**Experimental Procedures.**

**A**

**B**

Fig. 2. Tandem affinity purification of HBH-tagged Met4. The yeast transcription factor Met4 with a carboxyl-terminal HBH tag expressed from the chromosomal locus was sequentially purified by Ni^2+^ chelate chromatography and binding to streptavidin-agarose as described under “Experimental Procedures.” L, total cell lysates; FT, flow-through Ni^2+^-Sepharose; E/L, eluate from Ni^2+^-Sepharose, corresponding to the sample loaded on streptavidin-agarose; FTS, flow-through streptavidin-agarose. A, protein samples were separated by SDSPAGE and analyzed by Western blotting using an antibody against the RGSH6 epitope (left panel) or a streptavidin-HRP conjugate (right panel). Endogenous biotinylated proteins are much more abundant than the transcription factor Met4, and their detection with streptavidin-HRP thus produced the major bands as shown. Ubiquitinated forms of Met4 are indicated by asterisks. B, silver-stained gel of the purification samples indicating effectiveness of the second purification step. For silver staining, 40 times more sample from the Ni^2+^-Sepharose eluate and from the streptavidin-agarose flow-through were loaded as compared with total cell lysate and flow-through Ni^2+^-Sepharose samples.

din (K_d = 10^{-15} m) tolerates extremely stringent wash conditions such as 4% SDS, 8 m urea, organic solvents (e.g., 20% isopropanol/ethanol), and high salt conditions and thus guarantees highly purified samples (Ref. 17 and data not shown). The high affinity between biotin and streptavidin hampers efficient elution of HB-tagged proteins from streptavidin beads. This does not present a problem for mass spectrometric analyses because Lys-C and trypsin digestion is efficient on bead-bound proteins (data not shown and Refs. 18 and 19). Importantly, streptavidin itself proved to be largely resistant to Lys-C and trypsin digestion under these conditions. Typically less than 1% of the detected peptides resulted from fragmentation of streptavidin, which did not interfere with mass spectrometric analyses. For applications where elution of purified proteins is required, we inserted a TEV protease cleavage site that allows proteolytic release of the bound protein from streptavidin beads (Fig. 1A). In addition, we constructed additional derivatives of the HB tag that are useful for expression of amino-terminal or carboxyl-terminal fusion proteins (Fig. 1A). A second hexahistidine sequence was included to form the HBH tag, which increases efficiency of the first purification step by Ni^2+^ chelate chromatography and is particularly useful for purification of low abundant proteins.

We first tested expression and purification of the yeast transcriptional activator Met4 fused at its carboxyl terminus to the HBH tag (Met4^{HBH}). Met4 is posttranslationally modified by a ubiquitin chain, which is easily lost during native purification and is therefore well suited to test preservation of modifications during HB-based fractionation (20–22). Met4^{HBH} was expressed under control of its own promoter and, like most transcription factors, is of relatively low abundance. Cells were lysed in a buffer containing 8 m urea and incubated with a Ni^{2+} chelate resin (Fig. 2A). Detection of Met4^{HBH} with antibodies directed against the RGSH6 epitope, which is part of the HBH tag, showed efficient binding to the resin (Fig. 2A, left panel). Met4^{HBH} was subsequently eluted with a buffer containing 8 m urea and a combination of components that block the interaction of the hexahistidine sequence with the Ni^{2+} chelate resin (2% SDS, 10 mM EDTA, pH = 4.3). The pH of the eluate was readjusted to 7.5 and incubated with streptavidin-agarose. Close to 100% of Met4^{HBH} was retained on the streptavidin resin (Fig. 2A). Eukaryotic cells have between four and six endogenous biotinylated proteins (23). The first purification step by Ni^{2+} chelate chromatography efficiently removed these. This was evident by comparing load (L) and flow-through (FT) lanes in the Fig. 2A, right panel, where all biotinylated proteins were detected using a streptavidin-horseradish peroxidase (HRP) conjugate. Met4^{HBH} and its modified forms were the only biotinylated proteins that were retained on the Ni^{2+} chelate resin and thus specifically separated from endogenous biotinylated proteins (Fig. 2, right panel).

Importantly, immunoblot analysis of the purification steps with an antibody directed against the RGSH6 epitope (Fig. 2A, left panel) demonstrated greater than 90% in vivo biotinylation efficiency of the HBH tag because most of the Met4^{HBH} was retained on the streptavidin resin indicating the presence of biotin on Met4^{HBH} (Fig. 2A). Similar results were obtained with several other yeast proteins (Rpt1, Rpt5, Rpn11, Pre1, Pre10, Skp1, and Smt3), including proteins that were overexpressed from the GAL1 promoter (Met30, Cdc4, Met32, and Met31) and accumulated to significantly higher levels than Met4 (data not shown). In all cases in vivo biotinylation appeared to be close to 100%, demonstrating effective recruitment of endogenous biotin ligases by the HB tag.
Silver staining of the different purification fractions demonstrated the dramatic reduction of the background as compared with single step purification by Ni\(^{2+}\) chelate chromatography (Fig. 2B). Accordingly mass spectrometric analysis showed that nonspecific purification was reduced more than 6-fold (to 16%) by the second purification step on streptavidin beads as compared with the single step purification on Ni\(^{2+}\) chelate resin (data not shown). This represents a significant improvement of protein purification under denaturing conditions.

**HB Tag Is a Useful Tool for Proteome-wide Ubiquitin and Ubiquitin-like Protein Profiling**—Ubiquitin is a small 76-amino acid protein that is covalently attached to other proteins to regulate protein abundance and function (24). The important role ubiquitination plays in many biological processes has attracted proteome-wide approaches to better understand ubiquitin biology (25–27). One of the most promising approaches was pioneered by Peng et al. (28) in budding yeast. Hexahistidine-tagged ubiquitin was expressed in cells and, like endogenous ubiquitin, covalently attached in vivo to physiological target proteins by the cellular ubiquitin-conjugating system (28). Based on the hexahistidine-tagged ubiquitin, ubiquitinated proteins were purified and subsequently identified by mass spectrometry. Two other ubiquitin profiling experiments combined this approach with an initial cell fractionation step to isolate membrane-associated ubiquitination substrates (29) or with isolation of a subset of polyubiquitinated proteins on a polyubiquitin-binding affinity column (30). Immunopurification using antibodies directed against ubiquitin was also used in a large scale approach studying ubiquitination in human cells (31).

In general several aspects are important for global ubiquitin profiling experiments. First, ubiquitination is difficult to maintain during purification procedures unless highly denaturing conditions are used. This is mainly due to highly active deubiquitinating enzymes present in cell lysates. Second, co-purification of proteins that interact with ubiquitinated proteins, but are not themselves ubiquitinated, should be avoided. This can be achieved by denaturing fractionation conditions. Third, a two-step purification is desirable to reduce nonspecific background. Because ubiquitination profiling using HB-tagged ubiquitin fulfills all three criteria mentioned above, we expressed HB-tagged ubiquitin in yeast as well as HeLa cells to test the feasibility of this approach. Tagged ubiquitin competes with endogenous untagged ubiquitin for substrate conjugation. We therefore overexpressed HB-ubiquitin under control of the CUP1 promoter in yeast to achieve a high proportion of HB-ubiquitin in ubiquitinated proteins. We noticed that high level expression of HB-ubiquitin slowed down cell proliferation and reduced in vivo biotinylation efficiency. This was likely a result of depletion of cellular biotin levels because supplementation of the growth medium with 4 \(\mu\)M biotin could overcome these effects (data not shown and Fig. 3A). Biotin depletion in yeast was only observed under conditions where high levels of HB-tagged proteins were expressed. Expression of most HB-tagged proteins did not require biotin supplementation. Biotin depletion was also observed occasionally in mammalian cells and was manifested in reduced in vivo biotinylation efficiency, which we attributed to varying biotin concentrations in fetal bovine serum from different manufacturers or different lots. To eliminate this variability we routinely supplemented tissue culture media with 1 \(\mu\)M biotin.

HB-ubiquitin was efficiently attached to cellular proteins in yeast and mammalian cells as indicated by high molecular conjugates (Figs. 3 and 4B). To purify ubiquitinated proteins from yeast and mammalian cells we followed the strategy shown in Fig. 4A. Cells were lysed in a buffer containing 8 M urea, and lysates were incubated with a Ni\(^{2+}\) chelate resin and, after several wash steps, were eluted with a buffer containing 8 M urea, 2% SDS, 10 mM EDTA, pH 4.3 (Fig. 4B). The eluate was subsequently incubated with streptavidin-agarose (Fig. 4B). Silver staining of the samples from the purification steps showed the significant reduction of nonspecific background that was retained at the first fractionation step (Ni\(^{2+}\) chelate resin), demonstrating the improvement of this strategy compared with single step approaches (Fig. 4B).

Prior to mass spectrometric analyses, streptavidin beads were extensively washed with buffers containing 8 M urea, organic solvents, and SDS to remove nonspecifically bound proteins. Then on-bead tryptic digestion was performed, and the resulting peptides were extracted from the streptavidin-agarose, concentrated, and analyzed by one- and two-dimensional liquid chromatography tandem mass spectrometry. Ubiquitinated proteins from 200 ml of yeast cell culture were purified and analyzed. A total of 258 proteins were identified...
with high confidence (Supplemental Tables 1 and 2). 154 of them were identified by at least two independent peptides with both peptides having a peptide score of 25 or higher (Supplemental Table 1). The identified proteins fall into a variety of functional categories (Fig. 5).

Comparison of our data set with that obtained in previously described ubiquitin profiling experiments (28, 30) revealed significant overlap but also differences (Supplemental Tables 1 and 2). About 62% of the proteins identified in this study have been reported in previous large scale studies (28, 30). The additional proteins identified in our experiment might be a result of differences in cell culture conditions, purification strategies, and mass spectrometry platforms used.

We also analyzed our datasets to identify ubiquitin attachment sites as described by Peng et al. (28). The mass increase caused by two glycine residues that remain attached to the ubiquitinated lysine after tryptic digest is diagnostic for ubiquitination sites (25, 26). Applying this strategy, one can identify lysine residues in ubiquitinated proteins that were conjugated to ubiquitin. Furthermore different types of ubiquitin chain topologies can be distinguished because formation of ubiquitin chains requires isopeptide linkages between the carboxyl-terminal carboxyl group of a new ubiquitin molecule with the ε-amino group of one of seven lysine residues present in a substrate-attached ubiquitin molecule (Fig. 6). Consistent with a previous study (28), we found evidence for ubiquitin/ubiquitin linkages at all seven lysine residues of ubiquitin (Fig. 6). Peng et al. (28) found linkage through lysine

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**FIG. 4. Purification of ubiquitinated proteins.** A, schematic representation of the strategy. HB-tagged ubiquitin is expressed in cells and conjugated to target proteins in vivo by the cellular ubiquitination machinery. HB-ubiquitin competes with untagged endogenous ubiquitin in substrate conjugation, leading to mixed ubiquitin chains consisting of HB-tagged and untagged ubiquitin. Cell lysates are prepared under fully denaturing conditions, and ubiquitinated proteins are first fractionated by Ni²⁺ chelate chromatography and eluted with a low pH buffer containing 8 M urea, EDTA, and SDS. This step efficiently removes endogenous biotinylated proteins that would be purified in the next purification step. The eluate from the Ni²⁺-Sepharose is bound to streptavidin-agarose to further purify ubiquitinated proteins. After several stringent wash steps, samples bound to streptavidin beads can be digested with trypsin (‘on-bead digest’) to release peptides that are analyzed by LC MS/MS. B, lysates from yeast cells or mammalian cells expressing HBT-ubiquitin as described in Fig. 3, A and B, were fractionated by Ni²⁺ chelate chromatography and bound to streptavidin-agarose. All steps were carried out under fully denaturing conditions (8 M urea), L, total cell lysates; FT, flow-through Ni²⁺-Sepharose; E/LS, eluate from Ni²⁺-Sepharose, corresponding to the sample loaded on streptavidin-agarose; FTS, flow-through streptavidin-agarose. Protein samples were separated by SDS-PAGE and analyzed by Western blotting using an antibody against the RGSH₆ or streptavidin-HRP conjugate. Shown is the silver-stained gel (left lower panel) of the purification samples indicating effectiveness of the second purification step. For silver staining, 3 times more sample from the Ni²⁺-Sepharose eluate and from the streptavidin-agarose flow-through were loaded as compared with total cell lysate and flow-through Ni²⁺-Sepharose samples. U, ubiquitin; STRP, streptavidin.
29 only in combination with lysine 33. In contrast, we detected evidence for lysine 29 linkage independent of lysine 33 as well as in combination with lysine 33 (Fig. 6). We identified a total of three ubiquitin-derived peptides with two remaining diglycine signatures in the same peptide (Fig. 6). One of them has been reported previously (28). This strongly suggests the existence of forked ubiquitin chains in vivo.

Ubiquitin attachment sites also have been identified on several other proteins, including two subunits of the 20 S proteasome and ribosomal proteins (Fig. 6). Notably lysine 123 in histone H2B was identified as a ubiquitination site in our dataset (Fig. 6). Histone H2B is known to be monoubiquitinated on the lysine residue we identified (32). Thus, our strategy is effective in analyses of poly- as well as monoubiquitination.

**HB Tag Purification Combined with in Vivo Cross-linking for Mass Spectrometric Identification of Transient and Weak Protein Interactions**—Currently the most effective strategies to identify the protein interaction network involve tandem affinity purification under native conditions combined with LC MS/MS. Particularly the TAP tag, which consists of the immunoglobulin-interacting domain of Protein A and a calmodulin-binding peptide (4), has been very valuable in the analyses of protein complexes and has proven to be an important tool for proteomic studies in yeast and higher eukaryotes. However, many biologically important interactions between proteins are transient or relatively weak and are only poorly preserved during the two-step purification procedure because of the high dilution of the protein solutions in the later steps. Transient and weak protein interactions can be preserved by covalently linking protein complexes in vivo prior purification. We therefore combined in vivo cross-linking of protein complexes and HB tag-based two-step purification under denaturing conditions to facilitate the identification of protein complexes that are formed by less stable or transient interactions. The stringent purification conditions compatible with the HB tag are preferable as they remove non-cross-linked, interacting proteins, which might not mirror the in vivo composition of the protein complexes. Reduction of background is particularly important for the in vivo cross-linking approach because proteins cross-linked to proteins that are nonspecifically purified amplify the background. We tested this approach by purifying in vivo cross-linked Skp1 (Fig. 7). Skp1 is a core component of SCF-ubiquitin ligases and forms several distinct multiprotein complexes (33). A yeast strain expressing
HBH-tagged Skp1 from its native promoter was treated with the simple cross-linking reagent formaldehyde to covalently link proteins that are in physical contact. Lysates were prepared in a buffer containing 8M urea and sequentially purified on Ni²⁺/H⁺-Sepharose and streptavidin beads under fully denaturing conditions. After several stringent wash steps, peptides released from the streptavidin-agarose by digestion with trypsin were analyzed by mass spectrometry to identify interacting proteins. A number of known Skp1 interactors (34) were identified (Table I). In addition, proteins not connected previously with Skp1 were identified specifically in samples prepared from cells expressing Skp1HBH but not from cells expressing untagged Skp1 (Table I). It remains to be shown whether these interactions are biologically relevant. However, several proteins among the identified Skp1-interacting proteins have been linked previously to the ubiquitin/proteasome pathway, indicating a possible biological significance of these interactions because of the important role Skp1 plays in this pathway (33).

A strategy that combines HB tag-based purification under denaturing conditions with in vivo cross-linking and quantitative mass spectrometry has been applied to identify transient and weak interactors of the yeast 26 S proteasome (35). This approach, termed QTAX, led to the identification of several so far unknown proteasome-interacting proteins and underscored the potential of this approach that exploits stringent purification conditions compatible with the HB tag.

**DISCUSSION**

We developed a tandem affinity tag that is compatible with two-step purification under completely denaturing conditions...
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**Fig. 7.** Purification of *in vivo* cross-linked protein complexes. Yeast cells expressing carboxyl-terminal HBH-tagged Skp1 from the endogenous promoter were incubated with formaldehyde (1% final concentration) for 10 min at 30 °C. Cross-linking was quenched by addition of glycine (125 mM final concentration), and Skp1-HBH-bound proteins were purified under denaturing conditions on Ni²⁺-Sepharose and streptavidin-agarose. Protein complexes bound to streptavidin-agarose were eluted by digestion with trypsin and analyzed by 1-D MS/MS (see Table I). L, total cell lysates; FT, flow-through Ni²⁺-Sepharose; E/LS, eluate from Ni²⁺-Sepharose, corresponding to the sample loaded on streptavidin-agarose; FTS, flow-through streptavidin-agarose.

such as 8 M urea or 6 M guanidinium. These stringent conditions inhibit enzymatic activities during purification and guarantee preservation of protein modifications in the *in vivo* state. In addition, HB tag-based purification is compatible with cross-linker treatment and can thus be a useful tool to identify transient or low affinity protein interactions that are often lost during conventional purification strategies.

HB-tagged proteins are efficiently biotinylated *in vivo* in both yeast and mammalian cells by the endogenous biotin ligases. Biotin ligase activity does not appear to be limiting as high level expression of HB-tagged ubiquitin could not saturate *in vivo* biotinylation as long as growth media were supplemented with biotin (Fig. 3 and 4B). Although we tested HB tag-induced *in vivo* biotinylation only in yeast and mammalian cells, we expect that this approach should work well in most cells because biotin ligating systems are present in all organisms (23).

The HB tag induces addition of a biotin molecule to tagged proteins, which could theoretically interfere with protein function. We tagged a total of nine essential yeast proteins (Rpt1, Rpt5, Rpn11, Pre1, Pre10, Skp1, Smt3, Met30, and Cdc4) with the HB tag and some in mammalian cells and found generally no effect of the tag on protein function. Nevertheless the HB tag, like any other tag, can compromise function of some proteins. For example, we noticed phenotypic changes in yeast strains carrying a carboxyl-terminal HBH tag on the essential proteasome subunit Rpt1. However, similar phenotypic changes were observed with hemagglutinin-tagged Rpt1 (data not shown).

The strong affinity of biotin to streptavidin allows very stringent wash conditions to remove all non-covalently associated proteins. On the other hand, this strong affinity prevents efficient elution of purified proteins. For applications where elution of intact proteins is required, we included a TEV protease recognition site, which allows efficient elution from streptavidin beads by proteolytic cleavage (data not shown). TEV cleavage is not necessary if samples are analyzed by mass spectrometry because on-bead digestion with trypsin and/or Lys-C can be performed to release peptides for subsequent identification. Streptavidin is a protein, and hence there was initial concern that a large number of streptavidin-derived tryptic peptides would be generated by on-bead digestion and interfere with the mass spectrometry. However, it turned out that streptavidin is largely resistant to proteases. It is conceivable that the compact fold of streptavidin might limit protease access under the conditions used in our experiments. Consistent with previous reports, on-bead digestion with Lys-C and trypsin resulted in generation of only a small amount of streptavidin peptides, which did not interfere with mass spectrometry (18, 19).

*In vivo* cross-linking of protein complexes combined with mass spectrometric identification is an attractive strategy to identify transient or weak protein-protein interactions. The goal is to “freeze” protein complexes in their *in vivo* state and avoid any changes due to interactions that are lost during purification or form non-physiologically in the lysates. The challenging aspect of this approach is to minimize nonspecific binding to the affinity resins because cross-linking tremendously amplifies background due to stabilization of protein complexes that are interacting with nonspecifically purified background proteins. Formaldehyde *in vivo* cross-linking combined with immunopurification and mass spectrometric protein identification has been applied previously to detect low affinity protein interactions (36). Purified samples were separated by SDS-PAGE and analyzed by MS after in-gel digestion (36). The HB tag is compatible with purification of cross-linker-treated protein complexes, and the stringent purification and wash conditions help to reduce nonspecific binding to the affinity resins. Thus, samples can be analyzed directly by mass spectrometry without separation by SDS-PAGE. This should increase sensitivity of protein identification and lead to comprehensive description of protein complexes. Results from experiments that combine *in vivo* cross-linking with purification of HBH-tagged Skp1 demonstrated the feasibility of this approach. Several known as well as potential novel Skp1 interactors were identified. Nevertheless similar to other approaches using immunopurification of Skp1 complexes under native conditions (34) or systematic two-hybrid assays (37), some of the known Skp1-interacting proteins were not identified. This could be due to the relatively small amount of cell lysate we used in these experiments; it might have been insufficient to detect low abundance proteins. Furthermore the choice of cross-linking reagent might be crucial for the detection of some proteins. We used formaldehyde, which only cross-links lysine residues that are in very close proximity. Some interacting proteins might not have lysine residues positioned in that manner and will be lost during
denaturing purification. This can be overcome by using cross-linking reagents with longer spacer regions that can cross-link distant lysine residues. In addition, the use of fully reversible cross-linker might improve identification of low abundance interacting proteins by increasing the number of peptides that are useful for protein identification by mass spectrometry because cross-linked peptides cannot readily be used.

In vivo cross-linking combined with HB tag-based purification and quantitative mass spectrometric identification (referred to as QTAX) has been successfully applied to identify proteasome interactors (35). This study led to the identification of more than 40 new potential proteasome-binding proteins, which presumably are only transiently or weakly associated with the proteasome and have therefore been lost during conventional protein complex purification approaches.

The two-step purification under denaturing conditions seems particularly attractive to profile covalent modifications with ubiquitin and ubiquitin-like proteins. We generated ubiquitination profiles from yeast cells expressing HB-tagged ubiquitin. In addition to the identification of a large number of potential ubiquitination substrates, we also identified ubiquitin chain topologies and ubiquitin attachment sites. Consistent

| ORF      | Name                        | Comments                       |
|----------|-----------------------------|--------------------------------|
| YDR328C  | Skp1                        |                                |
| YOR133W  | Efi1a                       | Elongation factor 2            |
| YLR058C  | Shm2                        | Serine hydroxymethyltransferase|
| YJR033C  | Rav1a                       | Regulator of V-ATPase          |
| YAL005C  | Ssa1                        | Heat shock protein             |
| YLL024C  | Ssa2                        | Heat shock protein             |
| YER103W  | Ssa4                        | Heat shock protein             |
| YKL081W  | Tef4                        | Elongation factor 1-γ          |
| YML088W  | Ufo1a                       | F-box protein                  |
| YDR502C  | Sam2                        | S-Adenosylmethionine synthetase|
| YLL050C  | Cof1b                       | Cofilin                        |
| YKL152C  | Gpm1                        | Phosphoglycerate mutase        |
| YDL132W  |                             |                                |
| YNL311C  | Skp2a                       | F-box protein                  |
| YKR059W  | Tif1                        | Initiation factor 4A           |
| YBR196C  | Pgi1                        | Glucose-6-phosphate isomerase  |
| YEP062C/YIL053W | Gpp1/Gpp2c | 3-Phosphoglycerate synthetase 2 |
| YIL148W  | Rpl40A                      | 60 S ribosomal protein L40-A   |
| YER177W/YDR099W | Bmh1/Bmh2c | 14-3-3 protein                |
| YGR234W  | Yhb1                        | Flavohemoglobin                |
| YKL156W/YHR021C | Rps27A/Rps27Bc | 40 S ribosomal protein S27-A   |
| YNL121C  | Tom70                       | Translocaze mitochondrial      |
| YJR123W  | Rps5                        | 40 S ribosomal protein S5      |
| YLR167W  | Rps31                       | 40 S ribosomal protein S37     |
| YMR186W  | Hsc82                       | Molecular chaperone            |
| YBL030C  | Pet9                        | Mitochondrial ADP/ATP carrier  |
| YJR027W  | Ty1B                        | Transposon Ty1 protein B       |
| YMR046C  | Ty1A                        | Transposon Ty1 protein A       |
| YMR056C/YBR085W | Aac1/Aac3c | Mitochondrial ADP/ATP carrier  |
| YBR208C  | Dur1,2b                     | Urea amidolase                 |
| YJL034W  | Kar2                        | ATPase, protein import into the ER|
| YFL009W  | Cdc4a                       | F-box protein                  |
| YGR135W  | Cys4                        | Cystathionine β-synthase       |
| YPR181C  | Sec23                       | GTPase-activating protein      |
| YKL006W/YHL001W | Rpl14A/Rpl14Bc | 60 S ribosomal protein L14-A   |
| YBL072C  | Rps8A                       | 40 S ribosomal protein S8      |
| YDR037W  | Krs1b                       | Lysyl-IRNA synthetase, cytoplasmic |
| YKR014C  | Ypt52                       | GTP-binding protein            |
| YLR293C/YOR185C | Gsp1/Gsp2c | GTP-binding nuclear protein    |
| YJL204C  | Rcy1a                       | F-box protein                  |
| YBL076C  | Iis1b                       | Isoleucyl-IRNA synthetase      |

* a Proteins shown to interact with Skp1 (34, 40).
* b Proteins with connections to the ubiquitin/proteasome pathway (40).
* c Peptide sequence corresponding to either of these two proteins was identified.
with previous results (28), our data suggest that all seven lysine residues in ubiquitin can be used in vivo to form ubiquitin/ubiquitin linkages (Fig. 6). Surprisingly we identified three ubiquitin-specific peptides that indicated branching of the ubiquitin chain in vivo (Fig. 6). A physiological role of branched ubiquitin chains remains to be shown, but these results imply the possibility of a new layer of complexity in ubiquitin biology.

We also identified precise ubiquitin attachment sites in several ubiquitination substrates, including two subunits of the 20 S proteasome (Fig. 6). Notably lysine 123 in histone H2B was among the identified ubiquitin attachment sites we identified in this study. Lysine 123 is the known monoubiquitination site of H2B (32) demonstrating that our approach is compatible with analyses of monoubiquitination.

Mechanistically similar to ubiquitination, covalent attachment of other proteins such as SUMO, Nedd8, and ISG15 to target proteins has been recognized recently as an important regulatory mechanism for many biological processes (38, 39). HB-tagged versions of these ubiquitin-like proteins should be an effective tool for proteome-wide studies of their substrate proteins. Preliminary data using HB-tagged Smt3, the yeast homolog of SUMO, indicated that the strategy is readily applicable to other ubiquitin-like proteins.

Taken together, the HB tag and its derivatives are useful tools for proteomic studies using mass spectrometry. The tag allows tandem affinity purification under fully denaturing conditions and thus combines a high degree of purification with preservation of protein modifications in the in vivo status. In addition, the procedure is compatible with in vivo cross-linking, allowing identification of transiently associated proteins, and can thus be an important tool to probe and understand the dynamics of the proteome.

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