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Methods and Technical Advances

Tandem affinity purification in Drosophila

The advantages of the GS-TAP system

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Abbreviations: GS-TAP, protein G/streptavidin binding peptide tandem affinity purification; krz, kurtz; TEV, tobacco etch virus; yTAP, yeast tandem affinity purification

Key words: tandem affinity purification, GS-TAP, Drosophila, proteomics, kurtz, TEV, arrestin

Tandem affinity purification (TAP) has been widely used for the analysis of protein complexes. We investigated the parameters of the recently developed TAP method (GS-TAP) and its application in Drosophila. This new tag combination includes two Protein G modules and a streptavidin binding peptide (SBP), separated by one or two TEV protease cleavage sites. We made pMK33-based GS-TAP vectors to allow for generation of stable cell lines using hygromycin selection and inducible expression from a metallothionein promoter, as well as pUAST-based vectors that can be used for inducible expression in flies. Rescue experiments in flies demonstrated that the GS-TAP tag preserves the function of the tagged protein. We have done parallel purifications of proteins tagged with the new GS-TAP tag or with the conventional TAP tag (containing the Protein A and calmodulin binding peptide domains) at the amino terminus, using both cultured cells and embryos. A major difference between the two tags was in the levels of contaminating proteins, which were significantly lower in the GS-TAP purifications. The GS-TAP procedure also resulted in higher yield of the bait protein. Overall, GS-TAP is an improved method of protein complex purification because it provides a superior signal-to-noise ratio of the bait protein relative to contaminants in purified material.

Introduction

In the post-genomic era, analysis of the proteome is the next big challenge for molecular biology. A systematic study of protein complexes and protein networks has been made possible due to the advances in complex purification approaches and subsequent mass spectrometry based identification of components.[1] Among the available protein purification methods, the tandem affinity purification (TAP) procedure proves to be the method of choice because it provides both a high yield of the bait protein and low level of contaminants.[2] Different combinations of tags have been reported, but the TAP tag consisting of two Protein A modules, a TEV protease cleavage site, and a calmodulin binding peptide (hereafter referred to as the yTAP tag, "y" stands for "yeast") has been widely used both in focused small scale experiments as well as in large-scale proteomic surveys.[3-7]

We have previously reported that the yTAP tag can be successfully used to purify protein complexes from *Drosophila melanogaster* cultured cells and whole animals.[7] Recently, a new version of the TAP tag, containing two Protein G modules, a TEV protease cleavage site, and a streptavidin binding peptide (the GS-TAP tag) has been created and used in mammalian cell culture.[8] The authors reported an up to 10-fold improvement in yield with this new tag, compared to the yTAP tag.

We tested the applicability of the GS-TAP system in Drosophila and compared its performance in parallel experiments with the yTAP approach. To that end, we designed a set of vectors that allow inducible expression of GS-TAP tagged proteins in cultured cells and in vivo. Using these as well as previously described yTAP vectors, we created stable cell lines and fly lines expressing GS-TAP or yTAP tagged Kurtz (Krz), a Drosophila ortholog of mammalian β-arrestin proteins.[9,10] Krz is currently a focus of our efforts to understand the regulation of cell signaling in Drosophila. We found that addition of either tag does not alter the subcellular localization or function of Krz, and confirmed an improvement in the yield of the bait protein in the GS-TAP procedure in side-by-side comparisons with the yTAP tag. The most significant improvement, however, was observed in the level of contaminating proteins, which was markedly lower in the GS-TAP approach. We also show that the addition of the second TEV protease cleavage site results in a faster cleavage of the target. Taken together, these data demonstrate that the GS-TAP system represents a significant advance in protein purification methodology and can be employed to investigate the Drosophila proteome.

Results

New drosophila GS-TAP vectors. The new Drosophila GS-TAP vectors were developed on the basis of the pMK33 and pUAST vectors that we previously found to work well with the yTAP tag.[7] The pMK33-GS-TAP vectors are designed for either transient or

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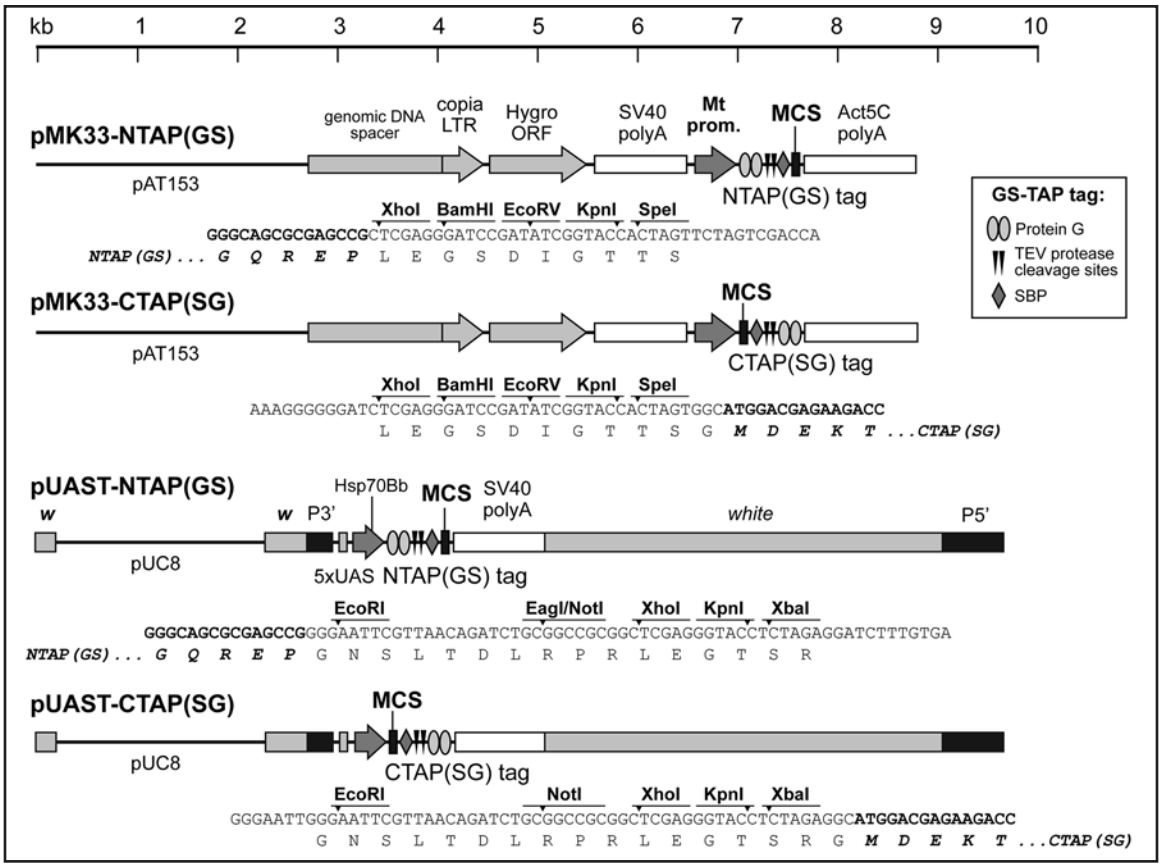


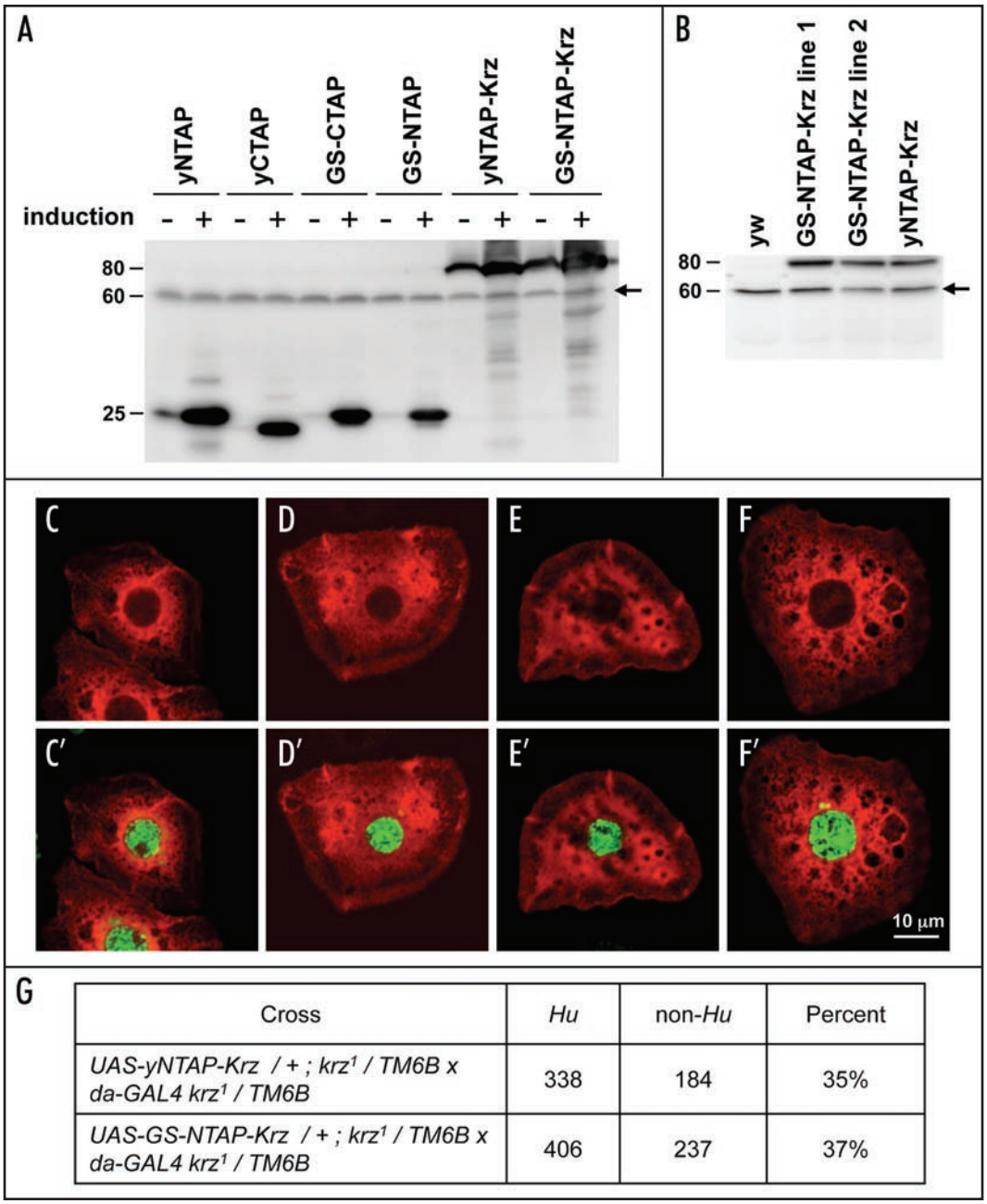
Figure 1. Drosophila GS-TAP vectors for inducible expression in cultured cells and transgenic flies. pMK33-NTAP(GS) and pMK33-CTAP(SG) vectors are designed for either transient or stable expression in Drosophila cultured cells. pUAST-NTAP(GS) and pUAST-CTAP(SG) vectors can be used for generating Drosophila transgenic lines and expression of tagged proteins from GAL4 drivers. Polylinker (MCS, multicloning site) sequences are listed under the vector diagrams, and unique restriction sites are indicated. The GS-TAP tag contains two Protein G domains, two TEV protease cleavage sites, and a streptavidin binding peptide (SBP). Complete vector sequences are available from Genbank: pMK33-NTAP(GS), EU663570; pMK33-CTAP(SG), EU663571; pUAST-NTAP(GS), EU663572; pUAST-CTAP(SG), EU663573.

stable expression of the GS-TAP tagged bait protein in Drosophila cultured cells (Fig. 1). Key features of these vectors include metallothionein promoter for copper-inducible expression of the tagged protein and a hygromycin resistance cassette, which eliminates the need to cotransfect a helper plasmid during creation of stable cell lines. The metallothionein promoter is advantageous because it avoids the potential toxicity of constitutively expressed proteins, and also allows control of expression level by varying copper sulfate concentration. The pUAST-GS-TAP vectors are based on the widely used pUAST vector that allows expression of the tagged proteins using a multitude of available GAL4 drivers (Fig. 1).[11] This vector can also be modified to include user-specific promoters, such as the *armadillo* promoter used in this study (see below).

Expression and functional characterization of the GS-TAP-Krz constructs. We began side-by-side comparisons between the yTAP and GS-TAP procedures by generating a matching set of constructs that contained full-length Krz open reading frame tagged at the amino terminus with either the yTAP or GS-TAP tags. Because the yTAP tag contains only a single TEV protease cleavage site, for comparison purposes our GS-TAP-Krz constructs were also made with a single TEV site in the GS-TAP tag, GS-NTAP-1xTEV. Note however that the final set of vectors shown in Figure 1 contains two TEV cleavage sites, as this results in more efficient cleavage by the TEV protease (Fig. 4). Stable S2 cell lines were selected as described in Materials and Methods using pMK33-based constructs. Using pUAST-based TAP vectors, we generated transgenic Drosophila lines carrying Krz fusion proteins tagged at the amino terminus with the yTAP or GS-TAP tags (collectively referred to as TAP-Krz). To facilitate large-scale collection of embryos, we also created Drosophila lines in which GS-TAP-Krz or yTAP-Krz were placed under the control of the ubiquitously expressed *armadillo* (*arm*) promoter.[12]

We first assessed expression levels of the TAP-Krz fusion proteins. Addition of copper sulfate to stable S2 cell lines resulted in similar expression levels of the empty tags and tagged Krz (Fig. 2A). The proteins were detectable even without induction, albeit at a lower level. In the embryos, the yTAP and GS-TAP tagged Krz fusion proteins were also expressed at similar levels under the control of the *arm* promoter (Fig. 2B). In these embryos, expression levels of TAP-Krz proteins were close to the level of the endogenous Krz protein (Fig. 2B, arrow). We note that the Protein A and Protein G domains present in the TAP tags cross-react with the detection antibodies used in Western blots. Because of this, the apparent levels of the TAP-tagged Krz proteins on Western blots are higher than the actual amounts.

To determine whether the addition of the GS-TAP tag would interfere with the proper subcellular localization of the Krz protein,



G

Cross	*Hu*	non-*Hu*	Percent
UAS-yNTAP-Krz / + ; krz¹ / TM6B x da-GAL4 krz¹ / TM6B	338	184	35%
UAS-GS-NTAP-Krz / + ; krz¹ / TM6B x da-GAL4 krz¹ / TM6B	406	237	37%

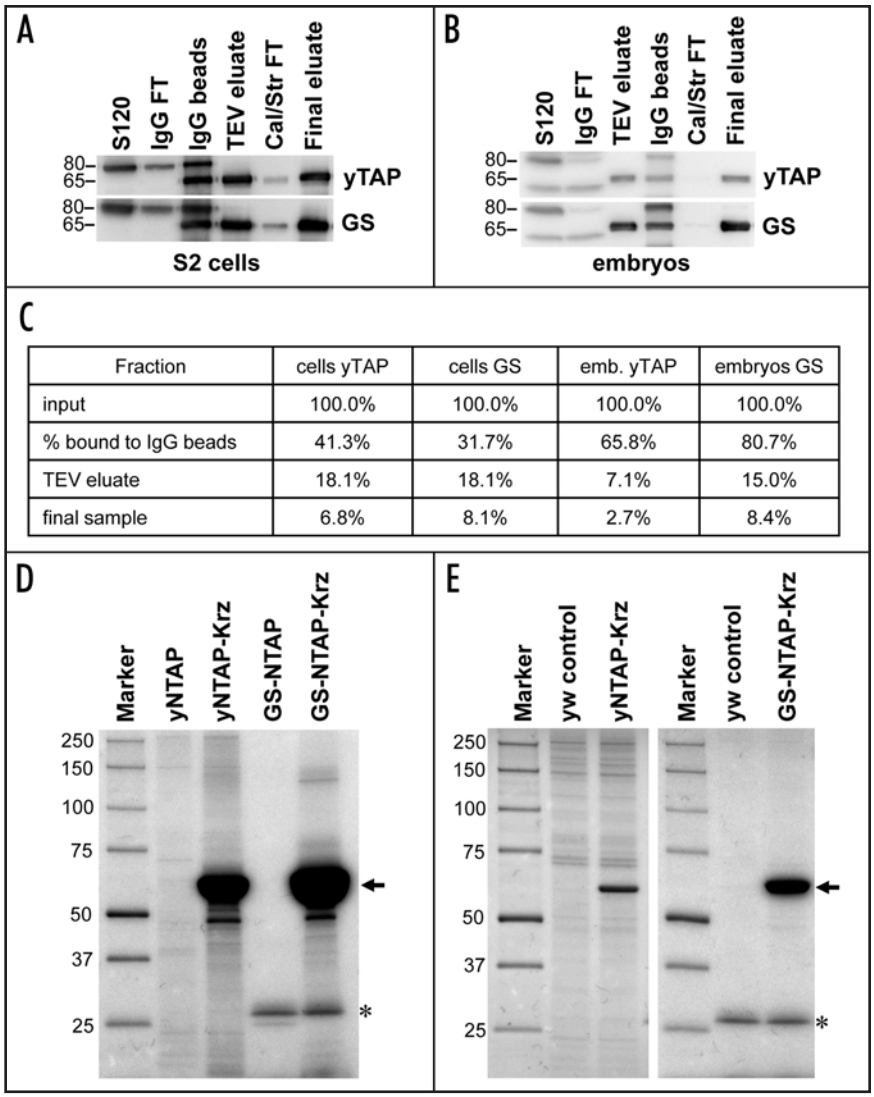
Figure 2. Validation of expression levels and functionality of the TAP-tagged Krz. (A) Expression of tags alone and TAP-tagged Krz in S2 cells stably transfected with indicated pMK33-based constructs. (B) Expression of TAP-tagged Krz in Drosophila embryos. Western blots in (A and B) were probed with pan-arrestin antibody (see Materials and Methods); arrows indicate endogenous Krz protein. (C–F′) Confocal images of S2 cells transiently transfected with pMK33-based Krz expression constructs. The Krz signal is red, DNA (DAPI stain) is green. (C and C′) HA-Krz; (D and D′) yNTAP-Krz; (E and E′) GS-NTAP-1xTEV-Krz; (F and F′) GS-NTAP-2xTEV-Krz. Scale bar, 10 μm. (G) Rescue of homozygous *krz*^{1} lethality with yNTAP-Krz and GS-NTAP-Krz transgenes. Parental genotypes are indicated, and the progeny was scored for the presence or absence of the *TM6B* balancer (*Hu* marker). Results shown are representative of several independent transgenic lines.

we transiently transfected S2 cells with Krz tagged at the amino terminus with the HA,[10] yTAP, GS-TAP-1xTEV or GS-TAP-2xTEV tags. All of these fusion proteins showed primarily diffuse cytoplasmic localization (Fig. 2C–F), consistent with previous reports.[10] Therefore, neither the yTAP nor GS-TAP tags altered the subcellular localization of the Krz protein.

To determine whether the GS-TAP tag was compatible with the normal function of the Krz protein, we crossed the *UAS-GS-TAP-Krz* lines with the ubiquitously expressed *da-GAL4* driver.[13] We found that this combination completely rescued the homozygous lethality of the strong *krz* loss of function allele, *krz*^{1} (Fig. 2G).[9] Viable offspring was obtained in ratios exceeding Mendelian expected values. Similar results were obtained with the *UAS-yNTAP-Krz* lines that were also expressed with the *da-GAL4* driver (Fig. 2G). Taken together, these results demonstrate that addition of the GS-TAP tag did not interfere with the normal function or localization of the Krz protein.

Use of the GS-TAP tag in tandem affinity purification results in higher yields and lower background, compared to the yTAP

tag. Protein extracts prepared from stable S2 cell lines and 0–6 hr Drosophila embryos expressing yTAP or GS-TAP tagged Krz were subjected to the tandem affinity purification procedure, as described in Materials and Methods. Analytical fractions were collected throughout and were analyzed by Western blotting and densitometry to quantify yield at each step (Fig. 3A and B). Quantification of the final TAP samples from embryo extracts showed a three-fold increase in the recovery of bait using GS-TAP compared to yTAP (8.4% vs. 2.7%, respectively; Fig. 3C). Analysis of different fractions showed that the increased yield in the GS-TAP procedure in the embryos was due to better binding to the IgG beads, more efficient TEV cleavage, and higher retention on the final beads. In contrast, purification profile from the GS-TAP procedure using S2 cells was comparable to the one obtained with the yTAP protocol, though the GS-TAP procedure again resulted in a somewhat higher final yield (8.1% vs. 6.8%, Fig. 3C). We hypothesize that the yield values were closer in the S2 cell experiment because the amount of the bait protein exceeded the column capacity. Consistent with this view, only 30–40% of the input protein was retained on IgG beads in the cell purifications, compared to 65–80% in the embryo extracts (Fig. 3C). Overall, final yield percentages from our GS-TAP samples were similar to those reported by Burckstummer et al.[8] (8.1% in the cell extracts and 8.4% in the embryo extracts), and were in both cases higher than the yTAP values.



Fraction	cells yTAP	cells GS	emb. yTAP	embryos GS
input	100.0%	100.0%	100.0%	100.0%
% bound to IgG beads	41.3%	31.7%	65.8%	80.7%
TEV eluate	18.1%	18.1%	7.1%	15.0%
final sample	6.8%	8.1%	2.7%	8.4%

Figure 3. Comparison of performance of the yTAP and GS-TAP approaches. (A and B) representative purification profiles from S2 cells (A) and whole embryos (B). S120, input extracts; IgG FT, flow-through after binding to IgG beads; IgG beads, material left on the IgG beads after TEV cleavage; TEV eluate, material eluted after TEV protease cleavage; Cal/Str FT, flow-through after Calmodulin (for yTAP) or Streptavidin (for GS-TAP) beads; Final eluate, final sample. Western blots were probed with pan-arrestin antibody. The following portions from each purification step were loaded on the gel: S120, 1/3000; IgG FT, 1/3000; IgG beads, 1/200; TEV eluate, 1/400; Cal/Str FT, 1/172; Final eluate, 1/160. (C) Quantitation of yield at different purification steps. Corresponding bands from (A and B) were measured by densitometry. All percentages are relative to the starting amounts. (D and E) Final samples were separated on gradient gels and stained with Colloidal Coomassie Blue. (D) Final fractions from S2 cell purifications. (E) Final fractions from embryo purifications. Arrows indicate the location of the tagged Krz protein, asterisks show streptavidin bands in the GS-TAP samples.

Final samples were separated by SDS-PAGE to further assess the yield and the load of contaminating proteins. We define contaminants as bands observed in control purifications in gels stained with colloidal Coomassie Blue. The overall yield of the tagged protein was in good agreement with densitometry values described above (Fig. 3D and E, arrow). Comparing the control samples, it became apparent that the GS-TAP procedure produced significantly less background in both cell extracts and embryos (Fig. 3D and E). The only visible contaminant in the GS-NTAP S2 cell extract final sample was streptavidin, which was eluted by SDS sample buffer from the streptavidin column (Fig. 3D, asterisk and data not shown). In comparison, the yNTAP S2 cell control sample contained many more contaminating proteins (Fig. 3D). An even more striking difference was observed when final samples from embryo controls were compared, again with streptavidin being the only visible band in the *yw* control sample subjected to the GS-TAP procedure (Fig. 3E, asterisk). Apart from the band corresponding to the bait protein (arrow), most of the other visible bands present in the yNTAP-Krz final sample were likely to be contaminants since they were also present in the *yw* embryo extracts that were subjected to the yTAP procedure (Fig. 3E, left). In contrast, several bands were visible in the GS-NTAP-Krz final sample that were absent from the *yw* control sample and may therefore correspond to proteins interacting with Krz (Fig. 3E, right). These experiments demonstrate that in addition to an increased yield, a major advantage of the GS-TAP procedure is a much cleaner background of contaminating proteins. These two properties combine to provide a superior signal-to-noise performance of the GS-TAP method, compared to the yTAP approach.

Addition of a second TEV protease cleavage site results in faster cleavage of the GS-TAP tag. We reasoned that the addition of an extra TEV cleavage site would reduce the time necessary to cleave the tagged protein off of the IgG beads. To test this hypothesis,

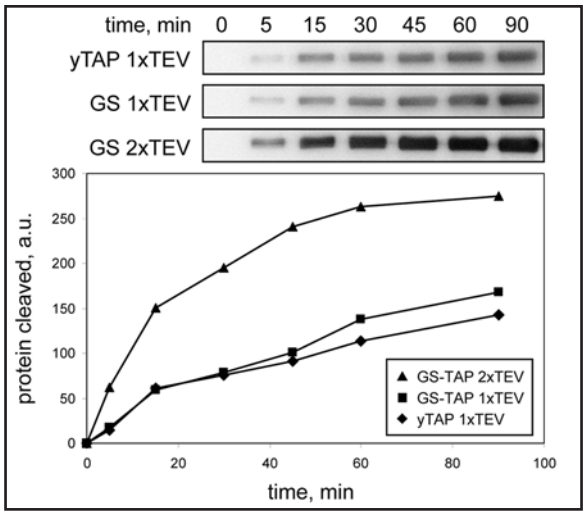


Figure 4. Addition of the second TEV protease site results in faster cleavage. S2 cells were transfected with identical amounts of pMK33-yNTAP-Krz, pMK33-GS-NTAP-1xTEV-Krz or pMK33-GS-NTAP-2xTEV-Krz, lysed, and the tagged proteins bound to IgG beads. TEV protease was added and samples were taken at indicated time points (for details, see Materials and Methods). Western blots were probed with pan-arrestin antibody. Bands were analyzed by densitometry and the results are shown on the graph. The GS-NTAP tag with two TEV protease sites was cleaved approximately twice as fast as either the yNTAP tag or the GS-NTAP tag with a single protease site. a.u., arbitrary units.

we created three pMK33-based constructs containing Krz tagged with the yNTAP tag (which has a single TEV protease cleavage site), the GS-NTAP tag containing a single TEV protease cleavage site (GS-NTAP-1xTEV), or the GS-NTAP tag with two tandem TEV protease cleavage sites between the Protein G and streptavidin binding peptide domains (GS-NTAP-2xTEV). Lysates from transiently transfected S2 cells expressing these fusion proteins were incubated with IgG beads, washed and subjected to cleavage with the TEV enzyme. Samples were taken at defined time intervals, and the amount of cleaved protein was analyzed by Western blotting and densitometry (see Materials and Methods). The yNTAP-Krz and GS-NTAP-1xTEV-Krz showed similar cleavage rates (Fig. 4). At every time point tested, there was approximately 2-fold more cleaved protein in the GS-NTAP-2xTEV sample, compared to either the yNTAP or GS-NTAP-1xTEV samples (Fig. 4). Addition of the second TEV protease cleavage site therefore results in a faster cleavage rate. This may allow improvements in the TAP procedure by shortening the cleavage step, reducing the amount of the protease required for efficient cleavage, and possibly reducing the required amount of starting material. Our final set of vectors therefore contains two TEV protease cleavage sites in both the NTAP(GS) and CTAP(SG) configurations (Fig. 1).

Discussion

Since the introduction of the original yTAP tag, creation of the GS-TAP tag represents a significant advance in protein purification methodology. The applicability of the GS-TAP method to protein complex purification and subsequent analysis of subunits by mass spectrometry was studied in detail by Burckstummer et al.[8] We extended the characterization of the GS-TAP based protein purification procedure by investigating important parameters of the method that have not yet been studied. We have shown that the GS-TAP method can be used with success to purify tagged proteins from Drosophila cultured cells and whole embryos. Genetic rescue experiments presented here demonstrate that the GS-TAP tag does not alter the function of the tagged protein, an important consideration for studying protein interactions. Analysis of purification profiles comparing the yields obtained with the yTAP or GS-TAP tagged Krz showed that the GS-TAP procedure results in higher final yields, which was especially clear in the embryo purifications. We observed an approximately 3-fold higher yield of the purified bait protein using the GS-TAP method in embryos, compared to the yTAP procedure. This value is lower than the maximal 10-fold increase reported in Burckstummer et al.[8] This difference can be due to the fact that a different protein was tagged in this study, or due to saturation of the capacity of the system with high amount of input material.

Perhaps the most important parameter revealed in this work is the difference in the amounts of contaminating proteins observed in yTAP vs. GS-TAP purifications. We found that the GS-TAP procedure results in an obviously lower load of contaminants compared to the yTAP method. This difference was clear in the in S2 cell samples but was even more striking in the embryo preparations. Since the first binding steps (IgG beads) are essentially identical between the two methods, it is likely that the cleaner background in GS-TAP purifications results from the use of the streptavidin binding peptide (SBP) in the GS-TAP tag. SBP is an artificially selected sequence with high affinity to streptavidin.[14] According to our results, the use of the streptavidin column in the second binding step pulls down substantially fewer contaminants, compared to the calmodulin column used for the yTAP tags. In addition, the SBP tagged proteins can be efficiently eluted from the streptavidin column with biotin,[8] which can further increase the specificity (T. Burckstummer, personal communication). Combined with a higher yield, the cleaner background obtained with the GS-TAP method clearly results in a better signal-to-noise performance. This property is especially relevant for studies aiming at identification of interacting subunits of protein complexes. We are currently analyzing Krz interacting proteins by mass spectrometry and will report these results elsewhere.

One of the limiting factors of the original yTAP method was the extent of release of the tagged protein from IgG beads after TEV protease cleavage. We reasoned that the addition of an extra TEV protease site to the GS-TAP tag would improve this step, and found experimentally that this is indeed the case. A second TEV site resulted in faster cleavage, but the two sites may also improve accessibility of the substrate for the enzyme, which would result in a more complete recovery of the tagged protein.

The advantages of the GS-TAP tag described here and in Burckstummer et al.[8] make it an ideal general method for purifying proteins and analyzing protein complexes in higher eukaryotes. The vectors developed in this study will facilitate the application of the GS-TAP approach to studying the Drosophila proteome.

Materials and Methods

Plasmid construction. The GS-TAP plasmids were obtained from Tilmann Burckstummer,[8] and the GS-TAP cassettes were amplified by PCR and inserted into pMK33 or pUAST vectors.[7] The CTAP(SG) version already contained two tandem TEV protease cleavage sites and was not altered. The NTAP(GS) version contained

a single TEV site and was used to generate GS-NTAP-1xTEV constructs described in this study. To create the GS-NTAP-2xTEV tag, the second TEV site was inserted as an oligonucleotide into a unique ClaI site in the GS-NTAP-1xTEV sequence. The final set of pMK33 and pUAST vectors contains two TEV sites both in the NTAP(GS) and CTAP(SG) configurations. The complete sequences of the vectors are available in Genbank (accession numbers EU663570-EU663573, see Fig. 1). To create *arm-yNTAP-Krz* and *arm-GS-NTAP-Krz*, UAS sequences were removed from the pUAST vector and replaced with the *arm* promoter which is ubiquitously expressed.[12] pMT-HA-Krz was described previously.[10]

Drosophila lines and cell culture. Transgenic Drosophila lines were established using standard techniques. The *yw* line was used as a control in purifications from embryos. S2 cells were maintained in Schneider's Drosophila Medium (Gibco) supplemented with 10% heat-inactivated Fetal Bovine Serum (Gibco). To establish stable cell lines, S2 cells were transfected with pMK33-based constructs using Effectene transfection reagent (Qiagen). After 48 hours of incubation with the transfection reagent, cells were maintained in complete media with 300 μg/ml hygromycin (Sigma). Stable cell lines were established approximately one month after transfection.

Western blotting and immunostaining. Samples for Western Blots were run on a 10% SDS gel and transferred to a PVDF membrane (BioRad) for 90 minutes. Membranes were blocked in TTBS (50 mM Tris pH 7.5, 0.1% Tween, 150 mM NaCl) with 5% dry milk and 2% IgG free BSA (Rockland). Membranes were incubated overnight with rabbit pan-arrestin primary antibody (Affinity BioReagents, 1:1000), which cross-reacts with Drosophila Krz. Donkey anti-Rabbit HRP secondary antibody (GE Healthcare) was used at 1:2000. Membranes were exposed using a Kodak Image Station 4000R with a chemiluminescence reagent (PerkinElmer).

For immunostaining, S2 cells were transfected and plated on concanavalin-coated cover slips. Cells were fixed in 4% formaldehyde (Polysciences), washed with PBST (phosphate buffered saline with 0.1% Tween-20) and incubated with anti-HA monoclonal antibody (Sigma) for HA-Krz and mouse IgG for TAP-Krz constructs. Mouse IgG binds to the Protein A or G domains in the TAP tags. Secondary antibody was goat anti-mouse Alexa555 (Invitrogen). DAPI was used to visualize the DNA. Images were acquired on a Zeiss LSM510 confocal microscope.

Tandem affinity purification. Tandem affinity purification was performed as previously described,[7,15] with a modification for the GS-TAP tag. Complete detailed protocols are available upon request. For cell cultures, stable cell lines were induced overnight with 0.07 mM $CuSO_4$. Approximately 1–2 x 10^9 S2 cells (1 L of culture volume) were pelleted at 500 g and washed three times with ice-cold phosphate buffered saline (PBS). Cells were homogenized on ice in five volumes of Lysis Buffer (50 mM Tris pH 7.5, 5% glycerol, 0.2% IGEPAL, 1.5 mM $MgCl_2$, 125 mM NaCl, 25 mM NaF, 1 mM Na_3VO_4, 1 mM DTT, 1 mM EDTA, and 2X Complete protease inhibitor, Roche). Lysates were cleared for 30 min at 27,000 g and stored at -80°C.

We found that 4–5 g of dechorionated embryos were more than sufficient for identifying bands of the bait protein in the final sample when staining with Colloidal Coomassie Blue (Sigma). Embryos were collected from population cages and dechorionated using 50% bleach. 5 g of dechorionated 0–6 hr embryos were washed and immediately homogenized on ice with Lysis Buffer, using a Wheaton dounce homogenizer with a tight pestle. After 15-min incubation on ice extracts were centrifuged for 30 min at 27,000 g and stored at -80°C.

For TAP, lysates were thawed and clarified by ultracentrifugation at 120,000 g for 30 min. Supernatants were then filtered through a 0.45 μm filter (Corning) to reduce clogging of the columns. Resulting filtrates were incubated with 200 μL of IgG beads at 4°C for two hours (rabbit IgG agarose, Sigma). Beads were loaded on Mobicol columns (MoBiTec) and washed with 10 ml of Lysis Buffer followed by 5 ml of TEV cleavage buffer (10 mM Tris pH 7.5, 100 mM NaCl, 0.1% IGEPAL, 1 mM DTT and 0.5 mM EDTA). TEV enzyme (AcTEV, Invitrogen) was added to the beads (final concentration 0.1 units/μL) and samples were shaken for 90 minutes at 16°C in a Thermomixer (Eppendorf). Eluted proteins were collected by centrifugation and added to 120 μL of either Calmodulin Sepharose 4B (Pharmacia) in the case of yTAP, or Streptavidin beads (Pierce) in the case of GS-TAP. yTAP samples were adjusted to 2 mM Ca^{2+}. Beads were rotated for 45 minutes at 4°C, loaded on Mobicol columns and washed with either 6 ml CBP Wash Buffer (yTAP: 10 mM Tris pH 7.5, 100 mM NaCl, 0.1% IGEPAL, 1 mM DTT, 1 mM MgOAc, 1 mM imidazole, 2 mM $CaCl_2$) or with 6 ml TEV cleavage buffer (GS-TAP). Final samples were eluted with 40 μL of 4xSDS sample buffer and resolved on 4–12% Novex NuPage protein gels (Invitrogen). Gels were fixed and stained with Colloidal Coomassie Brilliant Blue G (Sigma) and imaged on Kodak Image Station 4000R.

TEV cleavage assay. Drosophila S2 cells were transiently transfected with identical amounts of pMK33-yNTAP-Krz, pMK33-GS-NTAP-1xTEV-Krz or pMK33-GS-NTAP-2xTEV-Krz. Cells were pelleted and washed 3 times with PBS. Cells were lysed with 800 μL of Lysis Buffer (see above) by pipetting and incubated on ice for 15 minutes. Lysates were cleared by centrifugation for 20 min at 14,000 g, added to 30 μL packed IgG beads (Sigma) and rotated for 2 hrs at 4°C. IgG beads were then washed three times with 1 ml of TEV cleavage buffer (see above). TEV enzyme (0.05 units/μL final concentration) was added to IgG beads and samples were shaken in a Thermomixer (Eppendorf) at 4°C at 900 rpm. Samples of protein cleaved off from IgG beads were taken at 0, 5, 15, 30, 45, 60 and 90 minutes and analyzed by Western blotting.

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References

1. Kocher T, Superti-Furga G. Mass spectrometry-based functional proteomics: from molecular machines to protein networks. Nat Methods 2007; 4:807-15.
2. Bauer A, Kuster B. Affinity purification-mass spectrometry. Powerful tools for the characterization of protein complexes. Eur J Biochem 2003; 270:570-8.
3. Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin B. A generic protein purification method for protein complex characterization and proteome exploration. Nat Biotechnol 1999; 17:1030-2.
4. Bouwmeester T, Bauch A, Ruffner H, Angrand PO, Bergamini G, Croughton K, Cruciat C, Eberhard D, Gagneur J, Ghidelli S, Hopf C, Huhse B, Mangano R, Michon AM, Schirle M, Schlegl J, Schwab M, Stein MA, Bauer A, Casari G, Drewes G, Gavin AC, Jackson DB, Joberty G, Neubauer G, Rick J, Kuster B, Superti-Furga G. A physical and functional map of the human TNFα/NFκB signal transduction pathway. Nat Cell Biol 2004; 6:97-105.
5. Gavin AC, Bosche M, Krause R, Grandi P, Marzioch M, Bauer A, Schultz J, Rick JM, Michon AM, Cruciat CM, Remor M, Hofert C, Schelder M, Brajenovic M, Ruffner H, Merino A, Klein K, Hudak M, Dickson D, Rudi T, Gnau V, Bauch A, Bastuck S, Huhse B, Leutwein C, Heurtier MA, Copley RR, Edelmann A, Querfurth E, Rybin V, Drewes G, Raida M, Bouwmeester T, Bork P, Seraphin B, Kuster B, Neubauer G, Superti-Furga G. Functional organization of the yeast proteome by systematic analysis of protein complexes. Nature 2002; 415:141-7.

6. Gavin AC, Aloy P, Grandi P, Krause R, Boesche M, Marzioch M, Rau C, Jensen LJ, Bastuck S, Dumpelfeld B, Edelmann A, Heurtier MA, Hoffman V, Hoefert C, Klein K, Hudak M, Michon AM, Schelder M, Schirle M, Remor M, Rudi T, Hooper S, Bauer A, Bouwmeester T, Casari G, Drewes G, Neubauer G, Rick JM, Kuster B, Bork P, Russell RB, Superti-Furga G. Proteome survey reveals modularity of the yeast cell machinery. Nature 2006; 440:631-6.
7. Veraksa A, Bauer A, Artavanis-Tsakonas S. Analyzing protein complexes in Drosophila with tandem affinity purification-mass spectrometry. Dev Dyn 2005; 232:827-34.
8. Burckstummer T, Bennett KL, Preradovic A, Schutze G, Hantschel O, Superti-Furga G, Bauch A. An efficient tandem affinity purification procedure for interaction proteomics in mammalian cells. Nat Methods 2006; 3:1013-9.
9. Roman G, He J, Davis RL. *kurtz*, a novel nonvisual arrestin, is an essential neural gene in Drosophila. Genetics 2000; 155:1281-95.
10. Mukherjee A, Veraksa A, Bauer A, Rosse C, Camonis J, Artavanis-Tsakonas S. Regulation of Notch signalling by non-visual β-arrestin. Nat Cell Biol 2005; 7:1191-201.
11. Brand AH, Perrimon N. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 1993; 118:401-15.
12. Sanson B, White P, Vincent JP. Uncoupling cadherin-based adhesion from *wingless* signalling in Drosophila. Nature 1996; 383:627-30.
13. Wodarz A, Hinz U, Engelbert M, Knust E. Expression of crumbs confers apical character on plasma membrane domains of ectodermal epithelia of Drosophila. Cell 1995; 82:67-76.
14. Keefe AD, Wilson DS, Seelig B, Szostak JW. One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. Protein Expr Purif 2001; 23:440-6.
15. Burckstummer T, Bennett KL. Analysis of cellular protein complexes by affinity purification and mass spectrometry. In: O'Connor CD, ed. Proteomics: Methods Express. Bloxham: Scion Publishing Ltd., 2007.