Overexpression of TAP/p15 Heterodimers Bypasses Nuclear Retention and Stimulates Nuclear mRNA Export*

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Isabelle C. Braun, Andrea Herold, Michaela Rode, Elena Conti, and Elisa Izaurralde‡
From the European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, D-69117 Heidelberg, Germany

Human TAP and its yeast orthologue Mex67p are members of the multigene family of NXF proteins. A conserved feature of NXFs is a leucine-rich repeat domain (LRR) followed by a region related to the nuclear transport factor 2 (the NTF2-like domain). The NTF2-like domain of metazoan NXFs heterodimerizes with a protein known as p15 or NXT. A C-terminal region related to ubiquitin-associated domains (the UBA-like domain) is present in most, but not all NXF proteins. **Saccharomyces cerevisiae** Mex67p and **Caenorhabditis elegans** NXF1 are essential for the export of messenger RNA from the nucleus. Human TAP mediates the export of simian type D retroviral RNAs bearing the constitutive transport element, but the precise role of TAP and p15 in mRNA nuclear export has not yet been established. Here we show that overexpression of TAP/p15 heterodimers bypasses nuclear retention and stimulates the export of mRNAs that are otherwise exported inefficiently. This stimulation of mRNA export is strongly reduced by removing the UBA-like domain of TAP and abolished by deleting the LRR domain or the NTF2-like domain. Similar results are obtained when TAP/p15 heterodimers are directly tethered to the RNA export cargo. Our data indicate that formation of TAP/p15 heterodimers is required for TAP-mediated export of mRNA and show that the LRR domain of TAP plays an essential role in this process.

Metazoan TAP and its yeast orthologue Mex67p are members of an evolutionarily conserved protein family, the NXF family, implicated in the export of messenger RNA from the nucleus. **Saccharomyces cerevisiae** Mex67p and the **Caenorhabditis elegans** protein NXF1 are essential for the export of bulk polyadenylated RNAs to the cytoplasm (2, 3), whereas human TAP (also called Hs NXF1) has been directly implicated in the export of simian type D retroviral RNAs bearing the constitutive transport element (CTE)1 (4). In **Xenopus laevis** oocytes, titration of TAP with an excess of CTE RNA prevents cellular mRNAs from exiting the nucleus (4–6), strongly suggesting a role for TAP in mRNA nuclear export, but direct evidence has so far remained elusive.

Members of the NXF family of proteins have a conserved modular domain organization consisting of a non-canonical RNP-type RNA-binding domain (RBD), a leucine-rich repeat (LRR) domain, a middle region showing significant sequence similarity to nuclear transport factor 2 (the NTF2-like domain) and a C-terminal ubiquitin-associated (UBA)-like domain (Fig. 1 and Refs. 1, 7, and 8). The LRR and the NTF2-like domains are the most conserved features of NXF proteins, whereas the RBD and the C-terminal UBA-like domain are not always present in NXF proteins (1, 3).

The N-terminal half of TAP includes the LRR domain, the RBD, and a less conserved region upstream of the RBD (fragment 1–372, Fig. 1). This protein fragment exhibits general RNA binding affinity and mediates binding to several mRNA-associated proteins such as E1B-AP5 (9) and members of the Yra1p/REF protein family (10, 11). Furthermore, the RBD of TAP is required in cis to the LRR domain for specific binding to the CTE RNA (7). Hence, the RBD and the LRR domain are essential for TAP-mediated export of CTE-containing cargoes (1, 7, 12, 13). Mutations within the LRR domains of TAP and Mex67p have been reported to affect cellular mRNA export (11, 12), but these mutations involve residues that have important structural roles and their substitution probably results in nonspecific structural aberrations.

The NTF2-like domain of metazoan NXFs mediates binding to a protein known as p15 or NXT. p15 is also related to TAP2 (8, 15, 16) but unlike TAP2, which forms homodimers, p15 heterodimerizes with the NTF2-like domain of NXF proteins (1, 8). The human genome encodes at least two p15 homologues, p15-1 and p15-2, and both interact with TAP (1). The NTF2-like domain also occurs in **Schizosaccharomyces pombe** and **S. cerevisiae** Mex67p, although there is no obvious p15 homologue encoded by the yeast genome (8, 15, 16). In **S. cerevisiae** Mex67p, this domain is implicated in the interaction with a protein known as Mtr2p (14, 17). Prediction of Mtr2p secondary structure and the observation that co-expression of human TAP and p15 in **S. cerevisiae** partially restores growth of a strain carrying the otherwise lethal mex67/mtr2 double knockout, suggest that Mtr2p may be a p15 functional analogue (8, 16).

A C-terminal fragment of TAP, the NPC-binding domain (Fig. 1, fragment 508–619), mediates direct interactions with nucleoporins and is necessary and sufficient for the localization of TAP to the nuclear rim (1, 9, 18). This fragment comprises the entire UBA-like domain and part of the NTF2-like domain, but p15 binding by TAP is not required for its interaction with nucleoporins (9). The UBA-like domain on its own (fragment 567–619) is not sufficient to localize TAP at the nuclear rim in **vivo**, but single amino acid changes in a conserved loop of this domain (NWD at positions 593–595 in human TAP) severely
Expression of Recombinant Proteins—Glutathione S-transferase protein fusions were expressed in *E. coli* BL21(DE3) strains. *E. coli* M15[pREP4] strain was used for expressing proteins cloned into the pQE60zz vector. Recombinant proteins were purified as previously described (4). For oocyte injections recombinant proteins were dialyzed against 1.5 M phosphate-buffered saline supplemented with 10% glycerol.

**DNA Transfections and CAT Assays—** DNA transfections and CAT assays were performed essentially as described before (1), with the following modifications. Human 293 cells were transfected using Polyfect transfection reagent (Qiagen) according to the manufacturer’s instructions. The transfected DNA mixture consisted of 0.25 μg of the CAT reporter plasmid pCMV128, 0.5 μg of pEGFP-C1 plasmid encoding TAP or TAP mutants, and/or 0.5 μg of pEGFP-N3 plasmid encoding zzp15. Transfection efficiency was determined by including 0.5 μg of pH110 plasmid (Amersham Pharmacia Biotech), as β-galactosidase expression from this vector is not affected by TAP overexpression. The total amount of plasmid DNA transfected in each sample was held constant by adding the appropriate amount of the corresponding parental plasmids without insert, and was brought to a total of 2 μg by adding pBSSKII plasmid when necessary. When Rev-M10 fusions were tested, the transfected DNA mixture consisted of 0.25 μg of the CAT reporter plasmid pCMV128, 0.5 μg of plasmids pCMV-RevM10-TAP or pCMV-RevM10-p15, and/or 0.5 μg of plasmids pEGFP-C1-TAP or pEGFP-N3zzp15. Transfection efficiency was determined by including 0.5 μg of pH110 plasmid. Plasmids pCMV-Rev and pCMV-RevM10 were used as positive and negative controls, respectively. Cells were harvested 48 h after transfection and CAT activity was measured as described (24). Protein expression levels were analyzed by Western blot using anti-GFP or anti-HA antibodies.

**Xenopus Oocyte Microinjections—** All DNA templates for in vitro synthesis of labeled RNAs have been described. These were dihydrofolate reductase mRNA, U5ΔSm and U6Δas snRNAs, U6-CTE, and human initiator methionyl tRNA (6, 9). AdHML81, Fushi tarazu (Ftz), and β-globin cDNAs have been described (25, 26). Ftz-218 and β-globin-247 cDNAs were kindly provided by Hervé Le Hir (Brandeis University). Oocyte injections and analysis of microinjected RNA by denaturing gel electrophoresis and autoradiography were performed as described (6). Quantitation was done by FluorImager (Fuji FLA-2000). The concentration of recombinant proteins in the injected samples is indicated in the figure legends.

**RESULTS**

**Overexpression of TAP/p15 Heterodimers Promotes the Nuclear Exit of Inefficiently Spliced pre-mRNAs—** Previously, we reported an assay that allows quantifying TAP-mediated stimulation of RNA nuclear export in cultured cells (1). In this assay, a TAP protein expression vector is co-transfected with the chloramphenicol acetyltransferase (CAT) gene encoded by the reporter plasmid pDM138 (22). This plasmid harbors the CAT coding sequence inserted into an intron, which is not efficiently spliced (22). Cells transfected with this plasmid retain the unspliced pre-mRNA in the nucleus, yielding only trace levels of CAT enzyme activity (22). Expression of TAP/p15 heterodimers bypass nuclear retention and promotes the export of the inefficiently spliced pre-mRNA, resulting in a 14–16-fold increase in CAT activity (1). In this study, we improved the sensitivity and expanded the dynamic range of the assay by using the reporter plasmid pCMV128 (Fig. 2A). This plasmid is related to pDM138 but has a CMV promoter instead of an SV40 promoter (23). Consequently, the basal level of CAT enzyme activity in cells transfected with pCMV128 is 10-fold higher than in cells transfected with pDM138 (not shown). Furthermore, co-expression of TAP/p15 heterodimers with the reporter plasmid pCMV128 caused a 44-fold increase in CAT activity (Fig. 2B).

**Nuclease protection analysis confirmed that TAP/p15 heterodimers enhanced γ gene expression but allowed the unspliced transcripts to enter the cytoplasm (not shown). As reported (1), overexpression of TAP in the absence of exogenous p15 results in a significant but modest increase of CAT activity, although, the levels of expression of TAP were also reduced (Fig. 2, B and C). Moreover, overexpression of p15 in the absence of exogenous TAP or in the presence of deletion mutants...
Role of TAP Domains in mRNA Nuclear Export

FIG. 1. Domain organization of human TAP protein. The N-terminal domain of TAP (residues 1–372) includes the minimal CTE-binding fragment (residues 102–372) and exhibits general RNA binding affinity. This domain also binds to several mRNA-associated proteins such as E1B-AP5 and REF/Aly, and carries an NLS recognized by transportin (1, 7, 9, 10). The N-terminal domain consists of an RNP-type RNA-binding domain (yellow), a leucine-rich repeat domain (green), and a less conserved region upstream of the RBD (purple). The domain boundaries of the RBD and the LRRs are as defined in the crystal structure of these domains (7). The C-terminal half of TAP consists of an NTF2-like domain (red) and a UBA domain (cyan). The minimal TAP fragments sufficient for p15 or nucleoporin binding (9) are indicated. Numbers indicate the position in the amino acid sequence.

of TAP that cannot bind p15 (TAP ΔNTF2 and TAP 1–372) had no significant effect on CAT expression (Fig. 2B). These results indicate that formation of TAP/p15 heterodimers is required for TAP-dependent stimulation of cat gene expression. These results also indicate that in cells overexpressing TAP, p15 becomes limiting, and vice versa, and thus no large pools of free TAP or p15 exist in vivo.

The LRR and NTF2-like Domains of TAP Are Essential for Its Export Activity—Using pCMV128 as a reporter we have investigated the role of TAP domains in RNA export. Tested TAP mutants include a deletion of the first 60 amino acids (TAP-(61–619)) and deletions of the RBD, the LRR, the NTF2-like and the UBA-like domains (TAP ΔRBD, TAP ΔLRR, TAP ΔNTF2, and TAP ΔUBA) (Fig. 1 and Table I). TAP fragments lacking the entire C-terminal half (TAP-(1–372)) or the N-terminal half (TAP-(371–619)) were included as negative controls. All tested TAP mutants localize within the nucleus when expressed in HeLa cells, with the exception of TAP fragment 371–619, which distributes between the nucleus and cytoplasm (Ref. 9 and data not shown).

In the absence of exogenous p15, none of the TAP mutants stimulated significantly cat gene expression (Fig. 2B, white bars), but their expression levels were also reduced in comparison with that of TAP (Fig. 2C). In the presence of p15, TAP mutants that bind p15 were expressed at a steady-state level comparable with that of wild-type TAP (Fig. 2C). Nevertheless, when assayed for the ability to induce CAT expression from pCMV128, TAP ΔLRR was completely defective, whereas TAP-(61–619) and TAP ΔUBA exhibited low, but significant residual activity. In contrast, TAP ΔRBD retained 38% of the activity of wild-type TAP (Fig. 2B, Table I). TAP mutant ΔNTF2, which does not bind p15 (8, 9), failed to stimulate CAT activity (Fig. 2B), but the expression of this mutant protein was reduced in comparison with that of the wild-type control (Fig. 2C, lane 13 versus 5). We therefore generated a second mutant in the NTF2-like domain of TAP by deleting residues 381–503. Despite that TAP-(Δ381–503) does not bind p15, its expression at a steady-state level was comparable with that of TAP (not shown). However, this protein does not stimulate cat gene expression (Table I). These results provide strong support for the conclusion that formation of TAP/p15 heterodimers is required for TAP-mediated RNA export stimulation.

Since deletions of entire domains may affect multiple interactions or TAP folding, we tested the effect of introducing point mutations in the LRR (see below) and the UBA-like domains (Fig. 2B). The mutations targeted conserved residues exposed on the surface of the protein and were designed on the known three-dimensional structure of the LRR domain and the predicted structure of the UBA-like domain (7, 8). TAP mutants W594A and D595R have a single amino acid change in the conserved loop of the UBA-like domain and have impaired nucleoporin binding (1, 8). These mutants have an effect which is similar to removing the entire UBA-like domain (Fig. 2B), so we conclude that this domain is critical for TAP-dependent RNA nuclear export.

TAP/p15 Heterodimers Trigger Nuclear Export When Tethered to the RNA Export Cargo—Next, we tested the effect of tethering TAP/p15 heterodimers directly to the pCMV128 pre-mRNA. In this context, stimulation of RNA export by the various TAP mutants should be independent of their ability to bind RNA or RNA-associated proteins. TAP wild-type and mutants were fused to the C terminus of an HIV Rev protein defective in export (RevM10). RevM10 carries two point mutations in the nuclear export signal and, unlike wild-type Rev, cannot promote export of RNAs bearing the HIV Rev response element (RRE) (Ref. 27, reviewed in Ref. 28). However, RevM10 has an intact RRE-binding domain and can target the fusion protein to RRE-bearing RNAs (27–29). Vectors expressing RevM10 fusions were co-transfected into 293 cells with the CAT reporter plasmid pCMV128, which carries the RRE inserted in the intron (23). Expression of RevM10-TAP fusion moderately stimulated CAT activity, in contrast its co-expression with p15 increased CAT activity 250-fold (Fig. 3A). Similar results were recently reported by Guskik et al. (29). Conversely, tethering of p15 via the RevM10 protein had no significant effect on CAT activity, but its co-expression with TAP resulted in a 150-fold stimulation of cat gene expression (Fig. 3A). When neither TAP nor p15 were fused to RevM10 a 40-fold stimulation of CAT activity was measured, in agreement with data shown in Fig. 24. Thus, the higher CAT activity measured when either subunit of the TAP/p15 heterodimer was fused to RevM10 is likely to be due to its direct binding to the RRE.

Using this assay we then tested the effect of deleting individual TAP domains. Western blot analysis indicate that in the presence of p15, the expression levels of TAP mutants fused to RevM10 were comparable to that of the wild type control (not shown). When TAP was tethered to the RNA, removing the first 60 amino acids reduced its export activity by 2.5–3-fold (Fig. 3B and Table I). Unexpectedly, we found that deletion of the RBD increased the ability of RevM10-TAP fusion to promote cat gene expression. A possible explanation for this observation is that removing the RBD reduces the nonspecific binding of TAP to other RNAs, thereby increasing the pool of protein able to bind to the RRE-containing RNA. The export activity of RevM10-TAP was reduced by removing the LRR domain and completely abolished by deleting the NTF2-like or the UBA-like domains (Fig. 3B and Table I). In summary, when TAP is directly tethered to its cargo the RBD becomes dispensable for its export activity while the LRR, the NTF2-like domain, and the UBA-like domain still play a critical role. However, a protein fragment comprising these domains (TAP-(200–619)) exhibited 14% of the activity of wild type TAP, suggesting that residues upstream of the RBD are important for TAP function. This result is consistent with the observation that the first 60 amino acids of TAP, although not strictly necessary, contribute to its export activity (Figs. 2B and 3B).

Mutations in the LRR Domain Impair TAP-mediated RNA Export—The mechanism by which deletion of the LRR domain abolishes TAP function is unclear because TAP ΔLRR exhibits general RNA binding affinity, binds to REFs, E1BAP5, p15, and nucleoporins in vitro and localizes to the nuclear rim in vivo (not shown). LRR domains have a crescent shape with the convex surface formed by α-helices and the concave surface lined by β-strands (30). The concave β-sheet surface of LRR
domains has been proposed to mediate protein-protein interactions (30). At the concave face of the LRR domain of TAP, there is a conserved electronegative area defined by residues Asp\(^{228}\), Glu\(^{318}\), Glu\(^{319}\), Asp\(^{323}\), and Asp\(^{352}\) (7). Reverse-charge mutations of Asp\(^{228}\) (TAP D228K), which is conserved within the NXF family, and of Asp\(^{323}\) did not affect TAP-mediated expression of the cat gene (Table I). Asp\(^{352}\) plays a structural role and was not mutated (7). In contrast, reverse-charge mutations of residues Glu\(^{318}\) and Glu\(^{319}\) (TAP E318R,E319R) dramatically reduced stimulation of cat gene expression by TAP in the two

### Table I

| TAP Mutants + zzp15 | Relative Stimulation of Ad-mRNA Export in Oocytes | Relative Stimulation of CAT Expression in 293 Cells | Relative Stimulation of CAT Expression by RevM10 Fusions |
|--------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|
| TAP                | 100%                                         | 100%                                         | 100%                                         |
| TAP 61–619         | 19%                                          | 15%                                          | 39%                                          |
| TAP ΔRBD           | 75%                                          | 38%                                          | 240%                                         |
| TAP ΔLRR           | Inhibition                                   | 3%                                           | 27%                                          |
| TAP D228L (LRR)    | 91%                                          | 122%                                         | ND                                          |
| TAP D323L (LRR)    | 30%                                          | 3%                                           | 4%                                           |
| TAP D323K (LRR)    | ND                                           | 169%                                         | ND                                           |
| TAP ΔNTF2          | 6%                                           | 0.7%                                         | 0.4%                                         |
| TAP Δ381–503 (NTF2)| 3%                                           | 7%                                           | ND                                           |
| TAP ΔUBA           | 9%                                           | 13%                                          | 3%                                           |
| TAP W394A (UBA)    | 6%                                           | 13%                                          | 5%                                           |
| TAP D595R (UBA)    | 9%                                           | 3%                                           | ND                                           |

*ND, not determined.

### Fig. 2

The LRR and NTF2-like domains of TAP are essential for RNA export stimulation in 293 cells. A, schematic representation of the reporter gene encoded by the plasmid pCMV128 (23). SD and SA indicate the splice donor and acceptor sites of the intron. B, human 293 cells were transfected with a mixture of plasmids encoding β-Gal, CAT (pCMV128), and either GFP alone or fused N-terminal to TAP or various TAP mutants as indicated on the left. When indicated, a pEGFP-N3 derivative encoding zzp15 was co-transfected (+ p15, (black bars)). Cells were collected 48 h after transfection and β-Gal and CAT activity were determined. Data from three separate experiments are shown as fold activation of CAT activity relative to the activity measured when pCMV128 was co-transfected with parental plasmids without insert (−). The numbers are mean ± S.D. C, protein expression levels were analyzed by Western blot using anti-GFP antibodies. Lane 1 shows the untransfected control. The position of TAP mutants fused to GFP, of GFP itself, or of zzp15 is shown on the right.

### Fig. 3

TAP/p15 heterodimers promote export when tethered to RNA. A, human 293 cells were transfected with pCMV128, pCH110, and plasmids encoding RevM10 fusions of TAP or p15. When indicated, plasmids encoding GFP-TAP (+ Tat) or zzp15 (+ p15) were co-transfected (black bars). As controls, the reporter plasmids were co-transfected with empty vectors (−) or vectors expressing Rev or RevM10. Data from three separate experiments are shown as fold activation of CAT activity relative to the activity measured when GFP was coexpressed with pCMV128. The numbers are mean ± S.D. B, 293 cells were transfected with pCMV128, pCH110, zzp15, and plasmids encoding TAP or TAP mutants fused to RevM10. The stimulation of cat gene expression by TAP mutants in the presence of p15-1 measured in three independent experiments is expressed as percentage of the activity of RevM10-TAP.

Glu\(^{118}\), Glu\(^{119}\), Asp\(^{323}\) and Asp\(^{352}\) (7). Reverse-charge mutations of Asp\(^{228}\) (TAP D228K), which is conserved within the NXF family, and of Asp\(^{323}\) did not affect TAP-mediated expression of the cat gene (Table I). Asp\(^{352}\) plays a structural role and was not mutated (7). In contrast, reverse-charge mutations of residues Glu\(^{118}\) and Glu\(^{119}\) (TAP E318R,E319R) dramatically reduced stimulation of cat gene expression by TAP in the two
assays described above (Table 1). This mutant protein, how-
however, stimulated export of an excised intron lariat bearing the
CTE (7) and of an U6-CTE chimeric RNA (see below), indicat-
ing that it is properly folded. Thus, residues Glu318 and Glu319,
which are located at the C-terminal edge of the concave surface
of the LRR domain (opposite to Asp228), appear to be engaged in
interactions that are critical for TAP-mediated export of cellular
mRNA but not of CTE-bearing RNAs.

**TAP Stimulates the Export of mRNAs That Are Otherwise Inefficiently Exported**—To investigate whether TAP can di-
rectly stimulate export of cellular mRNA, Xenopus oocyte nu-
clei were co-injected with purified recombinant TAP and a mix-
ture of labeled RNAs. This mixture consisted of dihydrofolate reductase, β-globin, Ftz, and Ad-mRNAs, U535sm, and U644ss snRNAs, and a human initiator methionyl tRNA. The concentration of recombinant TAP and TAP mutants in the injected samples was 0.8 mg/ml and that of zzp15 was 2 mg/ml. B, purified recombinant TAP and TAP E318R, E319R were injected into Xenopus oocyte nuclei together with the mixture of radiolabeled RNAs described in panel A supplemented, with U6-CTE RNA. zzp15 was not included as it interferes with the export of U6-CTE RNA. In both panels, RNA samples from total oocytes (T), nuclear (N), and cytoplasmic (C) fractions were collected immediately after injection (t0: lanes 1–3, in both panels) or 90 min after injection, and analyzed on 8% acrylamide, 7 M urea denaturing gels. One oocyte equivalent of RNA, from a pool of 10 oocytes, was loaded per lane.

We also found that TAP directly stimulated export of other mRNAs. First, we decreased the export rate of β-globin and Ftz mRNAs by reducing the length of these transcripts from 360 and 343 nucleotides to 247 and 218 nucleotides, respectively (34). Fig. 4B shows that TAP stimulated the export of the shortened mRNAs (lanes 7–9). The effect of TAP was more dramatic on the export of Ad-mRNA and Ftz-218 mRNA, which are the least efficiently exported mRNAs. Second, we reduced the incubation time from 90 to 40 min so that less than 42% export was observed for all mRNAs tested (Fig. 4C, lanes 4–6). Under these conditions, p15 alone had no effect on export even though a 5-fold higher molar concentration was injected com-
pared with Fig. 4A (Fig. 4C, lanes 13–15). Coinjection of TAP, with or without p15, stimulated the export of all mRNAs, except dihydrofolate reductase mRNA (Fig. 4C, lanes 7–12). Again, the stimulatory effect of TAP was more dramatic on the export of mRNAs that were less efficiently exported, suggesting that TAP is limiting for these cargoes. TAP/p15 heterodimers had no significant effect on the export of β-globin, Ftz and Ad-mRNA produced by in vivo splicing of the corresponding pre-mRNAs (not shown).

**Role of TAP Domains in mRNA Export Stimulation in Xenopus Oocytes**—Next, we investigated the role of individual TAP
domains in mRNA export stimulation in *Xenopus* oocytes. The TAP mutants described above were expressed in *E. coli* and injected into *Xenopus* oocyte nuclei together with p15 and the mixture of labeled RNAs described in Fig. 4A. After a 90-min incubation period, 34% of dihydrofolate reductase and Ftz mRNAs and 48% of β-globin mRNA moved to the cytoplasm, while only 13% of Ad-mRNA was exported (Fig. 5A, lanes 4–6). Coinjection of full-length TAP resulted in a 4.2-fold stimulation of Ad-mRNA export (Fig. 5A, lanes 7–9, and Table I). TAP ΔRBD stimulated Ad-mRNA export by 3.4-fold (Fig. 5A, lanes 13–15). In contrast, deletion of either of the N-terminal 60 amino acids (TAP(61–619)), the LRR, the NTF2-like domain, or the UBA-like domain strongly reduced or abolished the export activity of TAP (Fig. 5A, lanes 10–12 and 16–24, Table I).

The effect of introducing point mutations in the LRR domain and the UBA-like domain of TAP was also tested. In the LRR domain only the reverse-charge mutation of residues Glu318 and Glu319 to Arg impaired stimulation of Ad-mRNA export by TAP (TAP E318R,E319R; Fig. 5B, lanes 10–12). In contrast, this mutant protein stimulated export of an U6-CTE chimeric RNA (Fig. 5B, lanes 10–12). TAP mutant D595R had the same effect as deleting the entire UBA-like domain (Fig. 5A, lanes 25–27 versus 22–24).

Because all TAP mutants that were impaired in mRNA export stimulation exhibited general RNA binding affinity in *vitro* (not shown), their absence of export activity cannot be attributed to a failure to bind RNA, but is likely to reflect impaired binding to p15 (for TAP ΔNTF2 and TAP Δ381–503) or to nucleoporins (for TAP ΔUBA and TAP Δ595R). It is currently unclear which interactions are affected by deleting the LRR domain or the first 60 amino acids of TAP. TAP(61–619) has a reduced *in vitro* affinity for E1B-AP5 (9), but the significance of this interaction for TAP function *in vivo* has not yet been established.

**DISCUSSION**

This study provides direct evidence for a role of TAP/p15 heterodimers in nuclear mRNA export. TAP/p15 heterodimers directly stimulate the export of mRNAs that are otherwise exported inefficiently. TAP/p15 heterodimers have no significant effect on the export of mRNAs that are efficiently exported or are produced by in *vivo* splicing of the corresponding pre-mRNA, suggesting that TAP/p15 heterodimers are not limiting for these cargoes. The observation that in *Xenopus* oocytes titration of TAP by an excess of CTE RNA inhibits mRNA nuclear export irrespective of whether or not the mRNA has been generated by splicing (4–6), however, suggests that TAP/p15 heterodimers participate in the export of both spliced and intronless mRNAs.

The role of TAP domains in RNA export was analyzed in cultured cells and in *Xenopus* oocytes. Despite the differences between these cell types and the export cargoes analyzed, the results obtained in these two systems are in surprisingly good agreement (Table I). These results indicate that TAP-mediated export of mRNA strictly requires the LRR and NTF2-like domains, whereas deletion of the first 60 residues of TAP or of the UBA-like domain strongly impairs TAP function. The functional importance of the LRR and NTF2-like domains is underscored by the observation that these domains are the most conserved among NXF proteins (1). Only the RBD is dispensable for mRNA export stimulation by TAP. Similar results were obtained by tethering TAP to the RNA via the RevM10 protein, although in this case TAP mutants lacking the first 60 amino acids or the LRRs exhibited 39 and 27% of the activity of wild type TAP, respectively. This suggests that these domains are crucial for cargo binding by TAP but can be deleted when TAP is directly tethered to its cargo (Fig. 3).

**Interaction of TAP with mRNP Export Cargoes**—The association of TAP with cellular mRNA may be direct or mediated by protein/protein interactions. Recently, several TAP partners that might facilitate TAP binding to cellular mRNA have been identified. These include E1B-AP5 (9), RAE1/Gle2 (9), and REF proteins (also called Yra in yeast and Aly in mice) (10, 11, 34). Apart from these, other RNA-binding proteins may act as adaptors between TAP and cellular mRNPs. In particular, the splicing coactivator SRm160, the acute myeloid leukemia-associated protein DEK, RNPS1 and Y14, together with REFs, are components of a 335-kDa protein complex deposited by the spliceosome 20–24 nucleotides upstream of a splice junction (25, 26). These proteins, either individually or as a complex, bind mRNA in a splicing-dependent, but sequence-independent way or may facilitate the recruitment of TAP to mRNA following splicing (26, 34–37). Therefore, TAP may not be limiting for spliced mRNAs because splicing guarantees the recruitment of TAP partners, and hence of TAP, in a sequence-independent manner. In contrast, the pCMV128 pre-mRNA and some intronless mRNAs, depending on their primary sequence and/or their length, may not be able to recruit TAP partners or TAP efficiently. Thus, TAP may be limiting for these particular cargoes so their export can be stimulated by TAP overexpression. Consistent with this, nuclear exit of inefficiently exported, intronless mRNAs can also be stimulated by microinjection of recombinant REFs in *Xenopus* oocytes (34).

An Essential Role for the LRR Domain of TAP in mRNA Nuclear Export—In this article, we have presented evidence that the LRR domain is essential for TAP function. Furthermore, we show that residues located at the C-terminal edge of the concave face of the LRR domain are critical for TAP-mediated export of cellular mRNA but not of CTE-bearing RNAs. The concave β-sheet surface of LRR domains has been proposed to mediate protein-protein interactions (30). Deletion of the entire LRR domain of TAP does not affect binding to its known partners (E1BAP5, REFs, p15, and nucleoporins).2 This suggests that the LRR domain of TAP binds one or more unidentified cellular ligands that are bypassed by the CTE, but is essential for export of cellular mRNA.

Formation of p15/TAP Heterodimers Is Required for TAP-mediated RNA Nuclear Export—The essential role of p15 in TAP-mediated RNA export was clearly demonstrated in cultured cells. Although co-expression of p15 increased the steady-state expression levels of TAP (Fig. 2C and Ref. 1), this effect was less dramatic than the stimulation of cat gene expression. For instance, the expression levels of GFP-TAP and RevM10-TAP were increased by a factor of 2–3-fold in the presence of p15, however, CAT activity was stimulated 40- and 250-fold, respectively, indicating that p15 not only stabilizes TAP but it is absolutely required for its export activity. The critical role of p15 in TAP-mediated export of intron-containing RNAs was also recently reported by Guzik et al. (29), although in this study a truncated form of TAP, (TAP(61–619)) was used.

In *Xenopus* oocytes, injection of TAP-Δ381–503 and TAP-ΔNTF2, which have no affinity for p15 (Refs. 8 and 9, this study), resulted in no export activity (Table I). Because in oocytes the recombinant proteins were stable (not shown), this suggests that TAP/p15 heterodimer formation is required for mRNA nuclear export. On the other hand, coinjection of p15 with TAP only slightly increased the mRNA export stimulation observed when TAP alone was injected, suggesting that p15 may not be limiting in the oocytes.

p15 has been implicated in export of tRNAs but also in

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CRM1-mediated export of U snRNAs and leucine-rich NESs (1, 16). In Xenopus oocytes, microinjection of p15 did not stimulate export of any of the RNA species tested (Figs. 4 and 5). Similarly, in cultured cells and in the absence of exogenous TAP, overexpression of p15 did not stimulate CAT expression even when it was tethered to the RRE-bearing pre-mRNA via RevM10 (Fig. 3). Moreover, co-expression of p15 with wild-type Rev did not significantly increase Rev-mediated export of RRE-containing pre-mRNAs (29). Together, these results suggest that p15 participates in mRNA export through its heterodimerization with TAP, or other members of the NXF family, and has no intrinsic export activity.

The UBA-like Domain of TAP Is Critical for Its Export Activity—The experiments described here indicate that the UBA-like domain contributes substantially to the export function of TAP, although a low but significant export activity was measured when this domain was deleted. Indeed, in the presence of p15, TAP ΔUBA and TAP W594A exhibited between 9 and 13% of the export activity of wild-type TAP (Table I). In S. cerevisiae, deletion of the UBA-like domain of Mex67p resulted in a thermosensitive growth phenotype and accumulation of polyadenylated RNAs within the nucleus, indicating that the UBA-like domain of Mex67p is required, but not essential for efficient mRNA nuclear export (14). In addition, Mex67p mutants lacking the UBA-like domain no longer localized to the nuclear rim (14). Overexpression of Mtr2p compensated for the lack of the UBA domain and restored growth, but the nuclear envelope localization of the protein. Similarly, NXF proteins lacking the UBA-like domain do not localize to the nuclear rim when coexpressed with p15 (1). Thus, in vivo Mex67p and metazoan NXF proteins lacking the UBA-like domain have a residual export activity in the presence of Mtr2p or p15 and may still be able to interact transiently with nucleoporins, although at equilibrium they are no longer localized at the nuclear rim. The NTF2-like domain might, therefore, mediate transient binding to nucleoporins when the UBA domain is not present thereby sustaining a residual export activity. Consistent with this hypothesis, it has recently been shown that high affinity interactions between nucleoporins and transport receptors are dispensable for NPC passage (40). For instance, NTF2 homodimers localize to the nuclear rim and facilitate the nuclear import of RanGDP, however, a point mutation that abolishes nucleorim localization (i.e. high affinity binding to the NPC) reduces but does not abolish the import function of NTF2 (40).

Distinct Requirements for TAP-mediated Export of Cellular and Viral mRNA—The results obtained in this study reveal different requirements for TAP-mediated export of cellular mRNA or of CTE-bearing RNAs. The RBd is dispensable for TAP-mediated export of mRNA but is essential for specific binding to the CTE RNA and therefore, for TAP-mediated export of CTE-containing cargoes (1, 7, 12, 13). Conversely, the first 60 amino acids of TAP have an important role in the stimulation of mRNA export, but are not required for TAP-mediated export of CTE-bearing RNAs (1, 9, 12, 13). Due to the lack of structural information, however, the role of these residues in mRNA nuclear export cannot currently be analyzed further. Interestingly, this domain is the least conserved among the NXF proteins (1). Our data suggest that the poor conservation of this domain does not reflect a non-essential function but may confer specific properties to the NXF proteins (i.e. substrate specificity or binding to specific partners).

The requirement of the NTF2-like and the UBA-like domains for CTE export are cargo-dependent (9). In Xenopus oocytes TAP-mediated export of CTE-bearing intron lariats is independent of these domains (9, 12) while export of U6-CTE requires the UBA-like domain but not the NTF2-like domain (9).

In quail cells, TAP-mediated export of an inefficiently spliced pre-mRNA carrying the CTE in the intron is abolished by mutations or deletions of the UBA-like domain (1, 19) but is only reduced by mutations preventing p15 binding (19).

Only the LRR domain is essential for export of both cellular mRNA and CTE-bearing RNAs, but its role in these processes is different. Indeed, reverse-charge mutations of Arg318 and Arg319 impaired mRNA export stimulation by TAP but supported CTE-dependent export (Ref. 7 and this study). Thus, it is likely that the mRNA export defect of this mutant is attributable to its inability to interact with some components of the nuclear export machinery that is required for mRNA nuclear export but bypassed by the CTE. This provides further support for the hypothesis that the mode of interaction of TAP with cellular mRNA is different from that with the CTE RNA and thus, that the CTE subverts TAP from its normal cellular function (7, 9, 12). Moreover, these results suggest that the assays described in this article are likely to reflect the genuine mRNA export activity of TAP.

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