The Avian Retroviral Receptor Tva Mediates the Uptake of Transcobalamin Bound Vitamin B₁₂ (Cobalamin)

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ABSTRACT The avian sarcoma and leukosis viruses (ASLVs) are important chicken pathogens. Some of the virus subgroups, including ASLV-A and K, utilize the Tva receptor for cell entrance. Though Tva was identified 3 decades ago, its physiological function remains unknown. Previously, we have noted an intriguing resemblance and orthology between the chicken gene coding for Tva and the human gene coding for CD320, a receptor involved in cellular uptake of transcobalamin (TC) in complex with vitamin B₁₂/cobalamin (Cbl). Here, we show that both the transmembrane and the glycosylphosphatidylinositol (GPI)-anchored form of Tva in the chicken cell line DF-1 promotes the uptake of Cbl with the help of expressed and purified chicken TC. The uptake of TC-Cbl complex was monitored using an isotope- or fluorophore-labeled Cbl. We show that (i) TC-Cbl is internalized in chicken cells, and (ii) the uptake is lower in the Tva-knockout cells and higher in Tva-overexpressing cells when compared with that of wild-type chicken cells. The relation between physiological function of Tva and its role in infection was elaborated by showing that infection with ASLV subgroups (targeting Tva) impairs the uptake of TC-Cbl, while this is not the case for cells infected with ASLV-B (not recognized by Tva). In addition, exposure of the cells to a high concentration of TC-Cbl alleviates the infection with Tva-dependent ASLV.

IMPORTANCE We demonstrate that the ASLV receptor Tva participates in the physiological uptake of TC-Cbl because the viral infection suppresses the uptake of Cbl and vice versa. Our results pave the road for future studies addressing the following issues: (i) whether a virus infection can be inhibited by TC-Cbl complexes *in vivo*, and (ii) whether any human virus employs the human TC-Cbl receptor CD320. In broader terms, our study sheds light on the intricate interplay between physiological roles of cellular receptors and their involvement in virus infection.

KEYWORDS avian leukosis virus, cobalamin, receptor, retrovirus

Retrovirus entry into host cells is a complex process orchestrated by the interaction of retroviral envelope (Env) proteins with receptor molecules on the cell surface. Avian sarcoma and leukosis virus (ASLV) is a widely studied species of the genus *Alpharetrovirus*. ASLV virions first attach to receptors via the surface (SU) subunit of Env, thus eliciting initial conformational changes in the metastable transmembrane (TM) subunit. The attached virions then undergo endocytosis, whereupon the low pH in the endosome triggers further TM conformational changes, leading to the fusion of viral and cellular membranes and the delivery of viral core into cytoplasm (reviewed in detail in references 1 and 2) (Fig. 1, left).

ASLV is classified into multiple subgroups, distinguished mainly by use of different receptors, immunogenicity, and the ability to interfere with one another. Although the

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FIG 1 Schematic representation of ASLV entry into chicken cells and TC-Cbl uptake in human cells. (Left) ASLV virions attach to cellular receptor Tva (an ortholog of mammalian CD320) through the trimeric envelope glycoprotein (Env), depicted as dark red knobs. This interaction primes the Envs and brings the virion into endosome. The acidic environment in endosomes triggers fusion of viral and cellular membranes (not shown), followed by delivery of viral core into the cytosol, where further replication steps ensue. (Right) Cbl is taken up by most cells when the vitamin is in complex with the high-affinity carrier protein, transcobalamin (TC). The TC-Cbl complex binds to the CD320 receptor and is endocytosed. After release in the endosome, Cbl is transported to the cytosol and further targeted to its metabolic functions.

ASLV Envs usually differ only within several small well-defined regions of SU (called hypervariable and variable), these differences still lead to interactions with completely different receptors (3). This is a unique phenomenon among retroviruses. ASLV subgroups A and K use the Tva (tumor virus A) receptor (4–6); the subgroups B, D, and E use the Tvb receptor (7, 8); and the subgroup C uses the Tvc receptor (9). ASLV subgroup J is a recombinant virus whose *env* gene arose by recombination with endogenous alpharetroviruses and which uses the Tvj receptor for entry (10, 11).

Tva belongs to the low-density lipoprotein receptor (LDLR) family and contains an LDL-A 40-amino acid (aa) domain. Tva exists in two isoforms, originating from the alternative splicing of exon 4 (4). One isoform is a type 1 transmembrane protein, whereas the second isoform is linked to the membrane surface via a glycosylphosphatidylinositol (GPI) anchor. The physiological function of Tva has remained unsolved. In 2004, we found that Tva (which resides on chicken chromosome 28) is orthologous to a mammalian gene, originally called 8D6A (12). This mammalian gene also belongs to the LDLR family and resides on human chromosome 19. Several years later, it was identified as a cellular receptor for the uptake of vitamin B₁₂ (cobalamin [Cbl]) from blood and renamed CD320 (13).

Cbl is an essential nutrient for all animals, including birds. It is produced by bacteria and mostly acquired through a diet consisting of animal products. Cbl deficiency in humans leads to hematological disorders (e.g., pernicious anemia) and/or neurological symptoms (for a review, see reference 14). After intestinal uptake, Cbl is secreted to blood and binds to the circulating transporter, transcobalamin (TC). The complex TC-Cbl is delivered to all tissues, binds to the membrane receptor CD320, and enters the cells (Fig. 1, right), where the liberated Cbl serves as a cofactor for two enzymatic reactions. Shortly after CD320 was identified as a TC-Cbl receptor, its knockout mice were generated and showed metabolic changes consistent with a moderate Cbl deficiency. This implied the existence of a parallel and CD320-independent cellular import of TC-Cbl (15, 16).

Based on the above, we explore a possible role of the avian receptor Tva in the recognition and the uptake of TC-Cbl, thereby testing a physiological relation between

FIG 2 Purification of TC. (A) Pairwise amino acid alignment of human and chicken TC. Alignment gaps are represented by dashes; bars, colons, and dots indicate identical, strongly similar, and weakly similar amino acid residues, respectively. The predicted signal peptide cleavage sites are marked by vertical arrows. (B) TC protein variants (human and chicken) used in this study with N- and C-terminal tags. The Strep tag sequences are highlighted in red. (C) Coomassie-stained SDS-PAGE of the purified TC variants. The constructs are labeled as in panel B. Molecular mass markers (kilodaltons, kDa) are shown on the left. h, human; c, chicken.

Tva and its human ortholog, CD320. In the present paper, we present a series of tissue culture experiments with Cbl tracers (labeled by ⁵⁷Co isotope or a fluorophore) and show that Tva does mediate the cellular uptake of TC-Cbl. Furthermore, we show that only ASLV subgroups, dependent on Tva for cellular entry, decrease TC-Cbl uptake in the infected chicken cells. The relation is reciprocal, and exposure of the cells to excessive TC-Cbl decreases the infection with Tva-dependent ASLVs.

RESULTS

Chicken and human TC. To use chicken TC (cTC) for our study of Tva-mediated uptake of TC-Cbl, we had to express and purify cTC. We used the predicted chicken sequence (cTCN gene; GenBank accession number [XM_015294930](#)) as a template for PCR to amplify the full cTCN coding region from chicken cDNA. The sequence that we obtained agreed with the predicted GenBank entry. The length of chicken and human TC proteins is 439 and 427 amino acids, respectively, and the pairwise identity/similarity is 35%/48% (Fig. 2A).

The cTCN coding sequence was then flanked by either Twin-Strep or Strep-His purification tag and placed at the 5'- or 3'-end, respectively, to produce the desired fusion proteins (Fig. 2B). Both constructs were heterologously expressed as secreted proteins in insect S2 cells and purified to near homogeneity by Strep-Tactin affinity chromatography with yields of 5 to 25 mg/liter of the medium. For a comparison, human TC (hTC) was cloned, expressed, and purified in an identical manner. The recombinant proteins migrated on SDS-PAGE as a single band of the expected size (Fig. 2C). Only results obtained with the 5'-tagged TCs are presented throughout this work. However, both

FIG 3 TC-[⁵⁷Co]Cbl uptake in chicken and human cells. The tracer was prepared by incubation of [⁵⁷Co]Cbl with hTC or cTC or without any carrier (as a negative control, see Materials and Methods). (A) The complex TC-[⁵⁷Co]Cbl was then added to chicken DF-1 cells in a serum-free medium. After 1 h of incubation at 37°C, the cell pellet was collected, and the internalized [⁵⁷Co]Cbl was determined in a gamma counter. (B) Comparable assays performed in human HEK293T cells. The means and standard deviations from three independent replicates are shown together with statistical significance (Welch's *t* test) indicated above the graphs. CPM, counts per minute.

5'- and 3'-tagged variants of cTC and hTC functioned similarly in the Cbl uptake assays (data not shown).

Labeling of TC with Cbl tracers. A Cbl molecule with a reporting group was used to monitor the cellular uptake of TC-Cbl complexes. Two types of tracers were used. Detection of commercially available [⁵⁷Co]Cbl was based on its radioactivity, while Cbl with attached fluorescein group (Cbl-F) provided fluorescent signal during microscopy. The latter conjugate was synthesized similarly to the previously reported method (17), with steps covering (i) functionalization of Cbl at its 5'OH ribose group, (ii) attachment of a diamine spacer to this position, and (iii) linking of *N*-hydroxysuccinimide (NHS)-activated fluorescein to the spacer (see Materials and Methods for further details). Binding and dissociation interactions of Cbl-F with hTC were examined via the respective amplification and decrease of fluorescence as described earlier for a similar ligand (17). The ratio of rate constants (dissociation/binding) pointed to a dissociation constant (*K_d*) of <1 pM. This result is approximate because the dissociation time interval of 1 h provided insignificant (<10%) chasing of Cbl-F from TC by the excessive CNCbl. Yet, a sufficient affinity of Cbl-F for TC was established in terms of our current assay.

TC-mediated Cbl uptake in chicken and human cell lines. We used the chicken cell line DF-1 and the human cell line HEK293T to study the uptake of free and TC-bound [⁵⁷Co]Cbl. The results are depicted in Fig. 3A and B. In both cell lines, significantly higher quantities of Cbl were internalized from its TC complex than from equimolar amounts of free Cbl. Interestingly, we observed a marked difference in the uptake of cTC-Cbl and hTC-Cbl, depending on the cells employed. The highest uptakes in chicken/human cells were recorded for cTC/hTC, respectively. Irrespective of species-specific differences, a significant uptake of Cbl was promoted by cTC in human cells and by hTC in chicken cells compared to the uptake of free Cbl. Accumulation of the latter was low and differed from the background only in the human cell line (presumably exposed to trace amounts of hTC from the medium, which can be produced by cultured cells [18]). We also determined that (i) the addition of an unspecific protein (bovine serum albumin [BSA]) did not enhance the [⁵⁷Co]Cbl uptake, and (ii) the TC-[⁵⁷Co]Cbl uptake was inhibited by the addition of 100-fold excess of unlabeled Cbl to TC prior to incubation with [⁵⁷Co]Cbl (data not shown).

Tva mediates TC-Cbl uptake in chicken cells. Next, we explored whether the Tva receptor is involved in the TC-mediated Cbl uptake in chicken cells.

We used three types of cells as follows: a wild type DF-1 cell line (DF-1), a DF-1 Tva-knockout cell clone (dTva) (19), and a DF-1 clone stably overexpressing Tva (oeTva). The wild-type DF-1 cells and the oeTva cells both express two forms of the receptor as follows: the transmembrane Tva-TM and the GPI-linked Tva-GPI.

First, we verified the cell surface expression of Tva in all three cell types. As Tva-specific antibodies are not available, this was done using the so-called immunoadhesin protein. The latter is composed of subgroup A SU (SU-A), fused to a constant region of a rabbit immunoglobulin (rlgG) heavy chain that specifically binds Tva (20, 21). As expected, the Tva-overexpressing cell clone showed a high Tva surface expression, wild-type cells showed moderate quantities of Tva, and the knockout clone did not reveal any distinctive expression (except for the background levels) (Fig. 4A).

We compared the cTC-mediated uptake of Cbl in the three types of cells, and the results are shown in Fig. 4B. Consistently with a role of Tva in TC-mediated Cbl uptake, we observed a remarkably high Cbl loading in the oeTva cells, followed by the wild-type DF-1 cells and the knockout dTva cells (all intergroup differences were significant). The uptake of TC-Cbl by dTva cells (significantly above the background) is in accord with the results obtained in mouse cells, where CD320 knockout does not completely block the TC-mediated Cbl import, implying a parallel transport (15, 16).

To further explore the intracellular location of Cbl, we used cTC with the bound fluorescent tracer Cbl-F, monitored by confocal microscopy. The cell membrane was additionally stained with CellBrite blue dye to better demarcate the internal cellular regions. The Tva-overexpressing clone showed a high amount of green signal inside the cells in accord with a high uptake of the TC-Cbl-F complex (Fig. 4C). In contrast, the wild type and the knockout cells both showed weak intracellular green signals, with only a small visible difference between them.

We examined TC-Cbl uptake via the transmembrane form (Tva-TM) or the GPI-linked form (Tva-GPI) taken separately. For this purpose, we constructed a distinct expression plasmid for each Tva isoform and transfected the plasmids into DF-1 Tva-knockout cells. Two days after transfection, we tested Tva expression (approximately 25% in both cases) and observed similar surface levels of Tva, determined by the mean fluorescence intensity (MFI) of Tva-positive cells (Fig. 4D). We also tested the uptake of TC-[⁵⁷Co]Cbl in cells transfected by either expression plasmid (Fig. 4E) and concluded that the two Tva isoforms can mediate TC-Cbl uptake. It seems, however, that Tva-GPI isoform is more effective as a TC-Cbl receptor because it showed a 2-fold higher uptake despite a similar surface exposure of Tva-GPI and Tva-TM.

Finally, we generated an expression plasmid, encoding the Tva-TM fused at the cytoplasmic terminus with the mCherry fluorescent protein. Transient transfection into the Tva-knockout DF-1 cells was documented by the red membrane staining from the mCherry fluorescence. After exposure of the cells to cTC-Cbl-F as described above, the transfected cells clearly showed a higher level of the green Cbl-F signal than the neighboring (Tva-negative) cells (Fig. 4F).

We conclude that TC-Cbl import into chicken cells is highly dependent on the levels of Tva receptor but that there may be an alternative pathway for TC-Cbl uptake in chicken cells, just like in mammals.

ASLV-induced block of TC-Cbl import. In general, viruses interact with their cognate receptors, which in turn may disrupt the receptors' physiological functions. For retroviruses, there is a well-described phenomenon of receptor interference (also called superinfection resistance), which occurs when newly synthesized Env proteins occupy receptors in infected cells. The mechanism is based on either a competitive block of the receptor's accessibility to viruses that use the same binding site or clearance of the receptors from the cell surface after formation of a receptor-Env complex (22).

We explored whether TC-Cbl uptake was altered in cells infected with viruses from the ASLV subgroups that employ Tva for cell entry. We infected DF-1 cells with ASLV-based replication-competent ASLV long-terminal repeat with a splice acceptor (RCAS)

FIG 4 Tva-mediated TC-Cbl uptake in chicken cells. (A) Surface expression of Tva was determined by incubation of the cells with the SUA-rlgG immunoadhesin, followed by Alexa Fluor 594-conjugated antibody specific for rabbit immunoglobulins. The cells were then analyzed by flow cytometry. A gate indicating percentage of highly Tva-positive cells is shown. Negative controls were done by omitting the SUA-rlgG step in the staining. An overlay of all three stainings is shown on the right. (B) TC-[^{57}Co]Cbl uptake assay was performed as described in the legend of Fig. 3. Results from three independent replicates are shown. Statistical notations are as in Fig. 3. DF-1, wild-type DF-1 cells; dTva, Tva knockout DF-1 cells; oeTva, DF-1 cells overexpressing Tva (see Materials and Methods for generation of the cells). (C) Fluorescence microscopy of TC-Cbl-F uptake (see Materials and Methods). The membranes were stained with CellBrite blue dye. In the composite confocal images, Cbl-F is shown as a green signal and CellBrite blue dye in blue. (D) Surface expression of Tva was determined by incubation of the transfected cells with the SUA-rlgG immunoadhesin, followed by Alexa Fluor 594-conjugated antibody specific for rabbit immunoglobulins. The flow cytometry is presented as histograms, with a gate of highly Tva-positive cells shown. For comparison, an overlay of all three histograms is depicted on the right. (E) The dTva DF-1 cells were transfected with Tva-TM or Tva-GPI expression plasmids as described in Materials (Continued on next page)

vector subgroups A, B, and K carrying a fluorescent marker gene (green fluorescent protein [GFP] or *Discosoma* species red fluorescent protein [dsRED]). The cells were passaged several times to allow the virus to spread throughout the culture. In the case of RCAS-A and RCAS-B, more than 99.8% of the cells were infected and dsRED positive (Fig. 5A). In the case of RCAS-K, ~80% of the cells were GFP positive. Based on our experience, we consider this observation to be an underestimate because GFP-lacking viruses gradually appear in the infected culture (6). We quantified the TC-[⁵⁷Co]Cbl uptake in each infected culture and compared it with the uptake in the uninfected DF-1 cells. The cultures, chronically infected with Tva-specific RCAS subgroups A and K, had decreased levels of TC-[⁵⁷Co]Cbl uptake, equivalent to approximately 50% of the uptake in our uninfected cells (Fig. 5B). Such decreased level is close to the Tva-independent import rate in the Tva-knockout cells (Fig. 4B). This fact suggests that most of the Tva-dependent uptake was blocked in the cells infected with RCAS-A or RCAS-K. In the cultures chronically infected with RCAS-B (targeting a different receptor [Tvb]), the TC-[⁵⁷Co]Cbl uptake was comparable to that of uninfected cells. Consequently, only ASLV subgroups, which use Tva for entry negatively affect the uptake of TC-Cbl.

TC-Cbl-induced suppression of ASLV infection. Finally, we examined the reverse situation. We pretreated DF-1 cells with a high (nonphysiological) amount of the TC-Cbl complex to see whether it would block subsequent infection by the three aforementioned ASLV subgroups. The DF-1 cells were pretreated with 400 nM cTC-Cbl, and after 30 min, they were infected using the three RCAS vectors. Two days later, the proportion of infected cells was determined by flow cytometry. The infection by subgroups A and K was significantly inhibited by the TC-Cbl pretreatment (Fig. 5C). Infection by subgroup B, which does not use Tva for entry, remained unaffected compared with that of the control RCAS-B-infected cells (pretreated with the basal medium only).

We conclude that infection of chicken cells by ASLV-A and ASLV-K impairs the uptake of TC-Cbl and that pretreatment of cells with TC-Cbl has the capacity to reduce ASLV infection. The results suggest either a competition for binding to the Tva receptor between the physiological ligand (TC-Cbl) and the ASLV Env or an indirect mechanism of Tva block.

DISCUSSION

In this work, we found that Tva serves as a TC-Cbl receptor in chicken cells, a finding consistent with the function of its mammalian ortholog CD320. Tva thus joins the group of retroviral receptors that also serve as transporters for B-family vitamins. Thus far, three such receptors have been identified, all members of a diverse group of solute carriers (SLC), as follows: first, the thiamine (vitamin B₁) transporter SLC19A2, which also serves as a receptor for koala retrovirus subgroup B (KoRV-B) and feline leukemia virus subgroup A (FeLV-A) (23–25); second, the riboflavin (vitamin B₂) transporter SLC5A1, also a receptor for porcine endogenous retrovirus subgroup A (PERV-A) (26); and third, the folate (vitamin B₉) transporter SLC19A1, which also mediates infection by GLN endogenous gammaretrovirus and a new FeLV variant (27, 28).

The overall physiological roles of ASLV receptors can be assessed (with some reservations) from their membership in relatively well-defined protein families. Such conjectures are applicable to Tvb (from the TNFR superfamily), Tvc (from the immunoglobulin superfamily), and Tvj (homologous to the human Na⁺/H⁺ exchanger). In contrast, the physiological role of Tva has, until now, remained enigmatic despite its identification as early as in 1993 (4, 5). Tva belongs to the LDLR family, a large group including proteins with very diverse functions (29). In 2004, we found that Tva is orthologous to a

FIG 4 Legend (Continued)

and Methods. As a control, pcDNA3 plasmid transfection was used (mock). Two days posttransfection, TC-[⁵⁷Co]Cbl uptake assay was performed as described in Fig. 3. Results from three independent replicates are shown. Statistical notations are as in Fig. 3. CPM, counts per minute. (F) Fluorescence microscopy analysis of TC-Cbl-F uptake using the Tva-mCherry expression construct. For details, see Materials and Methods. In the composite confocal images, CellBrite blue membrane dye is shown in blue, Cbl-F in green, and Tva-mCherry in red. The scale bars in microscopy images indicate 10 μm.

FIG 5 Functional interactions between ASLV infection and TC-Cbl uptake. (A) Establishment of chronically infected chicken DF-1 cells. The cells were infected with RCASBP(A)dsRed, RCASBP(B)dsRed, and RCASBP(K)GFP virus vectors (see Materials and Methods) at a multiplicity of infection (MOI) of approximately 1 and passaged several times to allow the virus to spread throughout the culture. The percentage of infected (fluorescence-positive) cells was determined by flow cytometry. (B) TC-[³⁷Co]Cbl uptake was quantified in the chronically infected cells as described in experiments presented in Fig. 3. CPM, counts per minute. (C) The preincubation of DF-1 cells with a high amount of chicken TC-Cbl (cTC-Cbl) (+) or with medium only (–) as a negative control. TC-Cbl-treated cells were infected with RCAS vectors of different envelope subgroups (MOI, between 0.1 and 0.4). Two days later, the percentage of infected cells was determined by flow cytometry. Results from three independent replicates are shown. All statistical notations are as in Fig. 3. ns, not significant.

mammalian gene, coding for a protein then called 8D6 antigen (8D6A) (12). First, 8D6A was considered a marker of follicular dendritic cells, and anti-8D6A antibodies hindered the development of lymphomas in mice (30–33). Later on, 8D6A was renamed CD320 and proved to be a TC-Cbl receptor (13). The earlier findings for lymphoma cells were assumed to be related to a decreased Cbl uptake and a hampered propagation of cells (caused by Cbl deficiency). Yet, this subject has never been reexamined.

Though TC-Cbl is a ligand for both Tva and CD320, the avian Tva differs from its mammalian CD320 orthologue in several ways. Tva contains one LDL-A domain, while mammals have two. Also, Tva exists in two isoforms (Tva-TM and Tva-GPI), while in mammals CD320 has only a transmembrane form. We have shown here that both Tva isoforms can mediate TC-Cbl import, the Tva-GPI isoform being slightly more efficient (Fig. 4E). Lim et al. (34) have shown that the two Tva isoforms also differ in virus internalization kinetics and subsequent stability in endosomes.

The residual TC-Cbl import in Tva-knockout cells points to the existence of a Tva-independent mechanism of Cbl uptake. Similarly, CD320-knockout mice remain viable and manifest only minor behavioral defects and a moderate Cbl deficiency (35–38), pointing to a parallel uptake of the vitamin.

Interestingly, several naturally occurring genetic defects in chicken Tva have been described. These range from missense mutation and splicing defects to the substitution of key amino acids in the LDL-A domain (12, 39). The phenotypic presentation in homozygotes comprises resistance to ASLV-A and no apparent effects on viability.

FIG 6 Comparison of the TC-Cbl uptake in an ASLV-infected and uninfected chicken cell. (Left) In the uninfected cell, TC-Cbl complex can be imported both through the Tva receptor and through an uncharacterized Tva-independent transport (denoted by question mark). (Right) In cells infected by ASLVs that employ Tva for entry (ASLV subgroups A and K), the Tva receptors are blocked by receptor interference. For simplicity, the newly synthesized Envs (dark red shapes) are shown to occupy the Tva receptors on the cell surface, although multiple mechanisms of receptor block have been described. Thus, the infected cells can utilize only the Tva-independent TC-Cbl import, resulting in lower import level.

Future studies are needed to explore the capacity of mutated Tvas to act as TC-Cbl receptors.

We can speculate whether Tva usage provides some evolutionary advantage for the compatible viruses in terms of tissue tropism or virus-induced signaling. ASLV subgroups A and B are by far the most common among the field virus isolates (40). Also, ASLV-K, which is emerging in Asia, appears to have independently evolved to use Tva for entry like ASLV-A does (6). All tissues (particularly those with a high rate of cell division) have a high demand in Cbl and express receptors for Cbl uptake. Relevantly, mammalian CD320 receptors are highly upregulated on the surface of tumor cells and metabolically active cells to meet the high demand for Cbl, especially for the purposes of nucleotide synthesis (41, 42). They are also upregulated in certain phases of the cell cycle (43, 44). The CD320 enrichment in tumors is so strong and specific that it has been exploited to visualize tumors using labeled Cbl derivatives (see reference 45 and references therein) and to target tumors for therapy with administration of toxic analogs (46–48). It will be interesting to determine whether Tva is also upregulated in chicken tumors. To the best of our knowledge, there is no data showing increased ASLV-A tropism for tumors or metabolically active cells. ASLV was described as favoring cells that undergo the S phase of the cell cycle (49–52). Conversely, ASLV-A can target even cells with extremely low amount of Tva receptors, especially through the Tva-TM isoform (53).

We have shown here that ASLV-A and ASLV-K can impair the TC-Cbl import in the infected cell (Fig. 6). Future studies are required to clarify whether Cbl metabolism is altered in infected chickens. The cytopathic effects of FeLV-A, which uses the vitamin B₁ receptor, are partly caused by vitamin B₁ deficiency and can be reversed by high doses of the vitamin (54). However, the existence of a Tva-independent uptake of TC-Cbl may also ensure a sufficient delivery of Cbl in the infected cells.

Finally, we have shown that the preincubation of cells with high nonphysiological concentrations of TC-Cbl (more than a 1,000-fold excess compared to human blood [55]) partially blocks ASLV infection (Fig. 5C). We did not assess if complete infection

block is possible because we were limited by the amount and concentration of the recombinant TC available. The incomplete blocking in our experiment can be explained by several scenarios as follows: (i) ASLV has a higher affinity for Tva and partially displaces TC-Cbl during the time of incubation; (ii) almost all of the Tva receptors are occupied by TC-Cbl, but the TC-Cbl and ASLV binding sites only partially overlap and do not guarantee mutually exclusive binding; or (iii) the two ligands do not compete directly for Tva binding but cause receptor internalization or redistribution, which only partially prevents the subsequent Tva interactions. Further research should specify the TC-Cbl binding sites on Tva and compare them with the well-defined ASLV-A binding determinants (56–58).

Our findings might also be of interest to those who deal with the biotechnological applications of Tva. For example, the Tva/RCAS system is widely used to generate transgenic mouse cancer models (59, 60). Also, Tva and Env-A-pseudotyped viruses have been used in cell fate tracing (61), the visualization of synaptic connections (62, 63), and the study of early retroviral uncoating steps (64).

In conclusion, our studies show that Tva has a physiological function in promoting cellular uptake of TC-Cbl in addition to its recognized role as a receptor for subgroups of ASLV. Thereby, Tva is added to the list of receptors involved in both the transport of vitamins and the binding of retroviruses. Further studies on the interplay between physiological roles of receptors and their roles in viral entry are needed in order to ensure a sufficient vitamin supply in spite of virus infection and, most importantly, to clarify to which extent a virus infection can be prevented by treatment with pharmacological doses of vitamins and/or its protein complexes.

MATERIALS AND METHODS

Heterologous expression and purification of TC and preparation of TC-[⁵⁷Co]Cbl. TC variants were heterologously expressed and purified in *Drosophila* Schneider S2 cells using protocols described previously (65). Briefly, expression plasmids encoding TC variants, together with pCoBLAST conferring blasticidin resistance, were transfected into *Drosophila* Schneider S2 cells using Effectene (Qiagen) according to the manufacturer's protocol. After 2 days, transfectants were selected by the addition of 40 μ g/ml blasticidin into the SFX serum-free medium supplemented with 10% fetal bovine serum (FBS). Three weeks posttransfection, the blasticidin-resistant S2 cell population was transferred into SFX medium, expanded, and expression of TC variants was induced by the addition of 0.7 mM CuSO₄ at a cell density of 1×10^6 /ml. Seven days postinduction, cells were harvested by centrifugation, and the conditioned media were concentrated by tangential flow filtration (TFF; Millipore) to 1/10 of the original volume. Concentrated media were supplemented with recombinant streptavidin (final concentration, 0.01 mg/ml) and loaded onto a 5-ml Strep-Tactin XT column (IBA) equilibrated in an equilibration buffer (100 mM Tris-HCl, 300 mM NaCl, pH 8.0). The column was washed with 50 ml of Tris-buffered saline (TBS), and the recombinant proteins were eluted with 5 mM biotin in the equilibration buffer. Pooled elution fractions containing purified TC variants were concentrated to 1 mg/ml by ultrafiltration, snap-frozen in liquid nitrogen, and stored at -80°C until further use. The typical yield of recombinant TCs was in the range 5 to 25 mg/liter of original culture with purity of >95%.

The TC-[⁵⁷Co]Cbl complex was prepared by incubation of 1.1 nM (approximately 100-fold excess compared to added Cbl) recombinant TC (chicken or human) with [⁵⁷Co]Cbl (MP Biomedical) in 4 ml Dulbecco's modified Eagle's medium (DMEM) (for each 10-cm culture plate) at room temperature for 1 h. An estimate of the [⁵⁷Co]Cbl concentration was available from the manufacturer, and according to this estimate, we used approximately 10 pM [⁵⁷Co]Cbl for the cell assays (see below).

Preparation of Cbl-F and TC-Cbl-F. The fluorescing conjugate of Cbl was prepared by a variant of a previously described procedure (17). Cbl was activated at the 5'OH group of ribose by incubating 40 μ mol CNCbl with 120 μ mol of 1,1'-carbonyl-di-(1,2,4-triazole) (CDT) in 0.6 ml of dimethyl sulfoxide (DMSO) (30 min, 30°C). Afterward, 200 μ mol of 4,7,10-trioxo-1,13-tridecanediamine was added, and the incubation was continued for 24 h at 30°C. The mixture was diluted to 30 ml with 0.05 M phosphate buffer (pH 6), and Cbl-spacer conjugate was extracted by shaking with 10 ml of phenol-chloroform mixture (1:1), separated as a bottom phase after a brief centrifugation. The following addition of chloroform, acetone, and water (10 ml each) transferred Cbl-spacer to the aqueous phase, used for purification. Positively charged amino group of Cbl-spacer bound to CM-Sepharose (30 ml, prewashed with 1 M phosphate, pH 6, and water), while unmodified Cbl was eluted with water. Cbl-spacer was eluted with 50 mM NaCl (the reaction yield was $\approx 25\%$). Cbl-spacer (10 μ mol in 10 ml of 0.05 M carbonate buffer, pH 9) was then interacted with 7 μ mol of 6-carboxyfluorescein succinimidyl ester (NHS-fluorescein ester; Lumiprobe, USA, Germany), dissolved in 0.6 ml DMSO. The reaction was conducted for 6 to 18 h in the darkness (no difference in the yield), whereupon the mixture was extracted by (i) phenol-chloroform and (ii) water. Complete separation of the produced Cbl-F from other reactants was achieved by either reverse-phase high-pressure liquid chromatography (HPLC) on C₁₈ column or adsorption on a small QAE

Sephadex A-25 column (2 ml, prewashed with 1 M NaCl and water), followed by elution of Cbl-F in 0.1 M ammonium acetate, pH 4.8, and lyophilization (yield of Cbl-F, $\approx 3 \mu\text{mol}$). All operations excluded exposure of fluorescein derivatives to a strong light to prevent their bleaching.

Interactions between Cbl-F and human TC were recorded by kinetic measurements of fluorescent response as described elsewhere for another fluorescing Cbl derivative (17), except for the excitation (495 nm) and emission (above 515 nm) used in the current setup. The binding assay monitored the increase in the fluorescent response over 0.5 s in the mixture of $0.5 \mu\text{M}$ TC + $0.5 \mu\text{M}$ Cbl-F, pH 7.5 (half-life $[t_{1/2}] \approx 0.05$ s). For an approximate assessment of the dissociation rate, decrease in fluorescence was followed over 1 h in the mixture of $0.5 \mu\text{M}$ TC-Cbl-F + $2 \mu\text{M}$ CNcbl, pH 7.5 (for further details, see reference 17).

To obtain TC loaded with Cbl-F for microscopy assays, TC (1 mg/ml) was mixed with $10\times$ molar excess of Cbl-F. The mixture was incubated for 30 min at room temperature and then loaded on a Superdex 10/30 HR75 size exclusion column equilibrated in 50 mM Tris-HCl and 140 mM NaCl, pH 7.4, to separate the TC-Cbl-F complex from free Cbl-F.

Cells and viruses. Chicken fibroblast cell line DF-1 and derived lines (Tva-knockout and Tva-overexpressing cells) were grown in a mixture of 2 parts DMEM and 1 part F-12 medium (Sigma-Aldrich) supplemented with 5% calf serum, 1% fetal calf serum, 1% chicken serum, streptomycin, and penicillin under 3% CO₂ atmosphere at 37°C. Human embryonic kidney cell line HEK293T was grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich) supplemented with 7% fetal calf serum, streptomycin, and penicillin under 5% CO₂ atmosphere at 37°C.

RCASBP(A)dsRed, RCASBP(B)dsRed, and RCASBP(K)GFP are replication-competent retroviral vectors based on ASLV (6, 66, 67) with envelope genes of subgroups A, B, and K, respectively. The vectors encode fluorescent reporter genes dsRed or GFP to enable detection of infected cells by flow cytometry. The vectors were transfected into DF-1 cells using Lipofectamine 3000 according to the manufacturer's protocol. The virus-containing media were harvested 9 days posttransfection, centrifuged at 2,000 g for 10 min to clear the cell debris, aliquoted, and stored at -80°C .

Plasmid expression constructs for Tva. Expression plasmids for both Tva isoforms (Tva-TM and Tva-GPI) were generated by PCR amplification from a chicken inbred line L15 cDNA (12). PCR was performed with primers TVAK2L (5'-CAGCTCGAGGTCGTCAT) and Tva-XbaI (5'-acgtctagaGGAAGGCCCTGCTCTATT), positioned in the 5' untranslated region (UTR) and 3' UTR of the Tva gene, respectively, which amplify both Tva isoforms. The capital letters in the primer sequences represent parts complementary to the Tva locus; lower-case letters indicate the XbaI restriction site (TCTAGA) and a 3-nucleotide sequence overhang included to ensure efficient cutting by the enzyme. In the TVAK2L primer, the XhoI site is underlined. After PCR amplification, the bands representing the two Tva isoforms were separated by agarose gel electrophoresis, excised, and purified using the QiaEX II gel extraction kit (Qiagen). Both fragments were digested with XhoI and XbaI restriction enzymes and subcloned in an XhoI/XbaI digested pcDNA3 vector. The resulting expression vectors were verified by Sanger sequencing.

The expression vector for production of Tva fused with mCherry fluorescent protein was generated using a previously published Tva expression vector pTvaS (12). This plasmid contains Tva-TM form truncated after the third amino acid (Lys114) of the cytoplasmic part and fused to hemagglutinin (HA) and 10-histidine (His) tags. The HA and His tags were replaced by mCherry coding sequence using NotI and SmaI restriction sites (mCherry was amplified with PCR primers 5'-AAAGGCGGCCGATCTTTGTGAGCAAGGGCGAGGAGATA and 5'-ATTCCCGGGGATCTCACTTGTACAGCTCGTCCATGCCG; NotI and SmaI are underlined).

Plasmid containing the Tva genomic clone was described previously (39). The genomic region was amplified from inbred chicken L15 genomic DNA with primers TVAK2L and TVAK3R (5'-GGAAGGCCCTGCTCTATTTC), subcloned into pGEM-T Easy vector (Promega), and then into pcDNA3 vector.

A plasmid vector for expression of cTC (amino acids 18 to 439) (GenBank accession number [XM_015294930](#)) in insect Schneider S2 cells was constructed by introducing a gene encoding cTCN lacking the native signal sequence between the BglII and NotI sites of modified pMT-BiP vectors (Thermo Fisher) featuring 5' and 3' sequences encoding TEV-cleavable Twin-Strep and Strep-His tags, respectively. cTCN was amplified from chicken cDNA using primers cTCN2ifSF (5'-CTCTGGCTCAAGATCTTGCGAAGCTCCAGCAGAGGC) and cTCN2ifSR (5'-TAGACTCGAGCGGCCGCTACCACTTGCTCAGGCGTAGG) and cloned by In-Fusion HD cloning kit (TaKaRa). A human TCN (hTCN) expression vector was constructed similarly, with PCR primers hTCN2ifSF (5'-CTCTGGCTCAAGATCTGAGATGTGTGAAATACCAGAGATGG) and hTCN2ifSR (5'-TAGACTCGAGCGGCCGCTACCACTGTAACAGCCTCAG) that amplify human TCN lacking the native signal sequence, and subcloned between the BglII and NotI sites of the pMT-BiP-SLIN vector.

Tva-overexpressing and Tva-knockout chicken cells. The chicken cells overexpressing Tva (oeTVA) were generated by stable transfection of DF-1 cells with Tva genomic plasmid with Lipofectamine 3000 (Invitrogen) according to manufacturer's instructions, followed by G418 selection. Selected cells were cloned by terminal dilution and tested for Tva surface expression with SUA-rlgG immunoadhesin. A cell clone with the highest Tva surface expression was selected for further experiments. Tva-knockout DF-1 cells have been described before (19), and they contain a homozygous 32-bp deletion in the second coding exon of the *tva* gene.

Cellular import assay for TC-[⁵⁷Co]Cbl. The protocol was adapted from previous publications, which analyzed the uptake in mammalian cells (68, 69). Cells (3×10^6) were seeded in 10-cm plates overnight. The next day, culture medium was removed, and a preformed TC-[⁵⁷Co]Cbl complex (~ 10 pM) or equimolar amounts of free [⁵⁷Co]Cbl were added in 4 ml of serum-free medium (DMEM). After 1 h incubation at 37°C, the cells were washed with phosphate-buffered saline (PBS), treated with trypsin-EDTA, and collected by centrifugation. The trypsin treatment also served to remove any TC-Cbl that remains

bound to the cell membrane. The radioactivity in the cell pellet was determined on the Wizard 3 gamma counter (Perkin Elmer). Counts were measured in a fixed 2-min period, and nonspecific (background) counts per minute (CPM) were subtracted. The values obtained served as a measure of TC-Cbl uptake, normalized for cell number (counted using Burkert's chamber).

Fluorescence microscopy analysis of TC-Cbl import. The cells were seeded on microscope cover glasses (1×10^5 to 1.5×10^5 cells on each cover glass). The next day, a fresh medium (including serum) was added, supplemented with the purified TC-Cbl-F complex (described above; concentration, 40 nM), and incubated for 1 h at 37°C. The cells were then fixed in 4% paraformaldehyde (PFA), stained with membrane dye CellBrite blue (Biotium) according to the manufacturer's protocol, mounted in PBS, and visualized using confocal microscope Leica TCS SP8 with 63 $\times$ /1.4 NA objective.

Flow cytometry analysis of Tva receptor expression. The SUA-rlgG immunoadhesin has been described previously (20). To produce the immunoadhesin protein-containing medium, DF-1 cells were transfected with the SUA-rlgG expression plasmid, and the medium was collected 2 days later and frozen at -80°C . To analyze the TVA surface expression, 1×10^6 cells were harvested with trypsin-EDTA, washed with staining solution (2% calf serum in PBS), and incubated with the SUA-rlgG immunoadhesin-containing medium for 30 min on ice. After 2 washes with the staining solution, the cells were incubated with goat anti-rabbit IgG linked to Alexa Fluor 594 (Invitrogen) diluted 1:1,000 in the staining solution on ice for 30 min in darkness. Then, the cells were washed with the staining solution, fixed in 0.5% PFA, and the fluorescence was quantified using an LSR II analyzer (Becton, Dickinson).

Infectivity assays. The DF-1 cells were seeded on a 96-well plate (1.5×10^4 cells per well). On the next day, the cells were incubated with 400 nM TC-Cbl complex (containing unlabeled cobalamin [Sigma; catalog number V6629]) in full medium for 30 min or with medium alone. The cells were then infected with RCAS vectors encoding fluorescent reporter proteins and bearing different envelope subgroup specificities (multiplicity of infection [MOI] between 0.1 and 0.4). After a 30-min incubation, the cells were washed twice with PBS, and a full medium was added. After 2 days, the cells were washed with PBS, trypsinized, and fixed in 4% PFA. The percentage of fluorescence-positive cells was determined by flow cytometry using the LSR II analyzer.

Statistical analysis. The statistical significance was evaluated using the Welch's *t* test. This test is robust to unequal standard deviations of the data sets tested.

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