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# VP1, the RNA-dependent RNA polymerase and genome-linked protein of infectious bursal disease virus, interacts with the carboxy-terminal domain of translational eukaryotic initiation factor 4AII

Brief Report

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**Summary.** Infectious bursal disease virus (IBDV), a member of the family *Birnaviridae*, is a non-enveloped, double-stranded RNA virus. Viral protein 1 (VP1), the putative RNA-dependent RNA polymerase, occurs in virions both as a free polypeptide and as a genome-linked protein, called VPg. To gain more insight in its function, we initiated a yeast two-hybrid screen. With this approach we identified the carboxy-terminal domain of eukaryotic translation initiation factor 4AII (eIF4AII) as an interactor for VP1. The association between these molecules was confirmed by co-immunoprecipitation analyses. eIF4A plays an essential role in the initiation of translation of both capped and uncapped mRNAs. Its association with IBDV VP1 suggests an involvement of this viral protein in IBDV mRNA translation. An interaction between VP1 and full-length eIF4AII was, however, not observed. In view of the known two-domain structure of eIF4AII it is conceivable that the interaction of VP1 with full-length eIF4AII requires collaborating proteins that open up its structure and expose the VP1-binding site in the carboxy-terminal domain. The biological relevance of the potential VP1-eIF4AII interaction is discussed.

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Infectious bursal disease virus (IBDV) is the causative agent of a highly contagious disease of young chickens that causes significant losses to the poultry industry. In the infected animal, IBDV targets the developing B lymphocytes in the bursa of Fabricius, in which it multiplies rapidly, leading to immune suppression. This condition increases the animal's susceptibility to opportunistic infections with other pathogens and reduces the growth rate of surviving animals [22].

IBDV belongs to the genus *Avibirnavirus* of the family *Birnaviridae* [16]. The virus consists of a non-enveloped capsid, 60 nm in diameter, that contains two segments of double-stranded RNA (dsRNA) of 3.3 kb (segment A) and 2.9 kb (segment B). Segment A contains two partially overlapping open reading frames (ORFs). The smaller ORF, located at the 5′ end, encodes VP5. This 17 kDa non-structural protein is not required for virus replication but plays a role in viral pathogenesis [40, 41]. The large ORF encodes a 110 kDa polyprotein precursor that is autocatalytically cleaved to produce pVP2 (48 kDa), VP3 (32 kDa), and VP4 (28 kDa). VP4 is the protease responsible for the processing of the polyprotein. During virion maturation pVP2 is further processed into VP2 (40 kDa) [13]. VP2 and VP3 are the structural proteins that constitute the viral capsid.

Segment B contains one ORF which encodes VP1. This 90 kDa protein shares a number of primary sequence features with RNA polymerases from diverse origins [7]. Based on its size, low copy number in virions, and the presence of several conserved domains associated with RNA-dependent RNA polymerases (RdRp) of other RNA viruses, VP1 is believed to be the virion-associated RdRp [16, 17, 20, 34]. Direct evidence demonstrating its RNA polymerase activity is, however, as yet lacking. VP1 interacts with VP3, an association likely to be essential for IBDV particle morphogenesis [10, 31, 36, 49, 50]. VP1 is present within virions both as a free polypeptide and as a genome-linked protein, called VPg, attached to the 5′ end of the positive strands of both genomic segments by a serine-5′ GMP phosphodiester bond [27, 39]. This bond can be formed *in vitro* during self-guanylylation of VP1, yielding VP1pG [14]. The VP1pG complex acts as a primer during RNA synthesis/transcription both *in vitro* [15] and *in vivo* [35], and the VP1 polypeptide remains covalently linked to the 5′-end of the RNA and thus becomes the genome-linked protein VPg. This suggests that the VP1-primed transcription reaction proceeds via an asymmetric, semiconservative, strand-displacement mechanism [15, 48]. Compared to other VPg's (e.g. adeno- and picornavirus) the IBDV VP1 protein is unusually large and its function as primer as well as polymerase is without precedent. RNA polymerase activity can be demonstrated in purified IBDV virions without any pretreatment [48] indicating that, like with reovirus [47], transcription can be initiated without the need for uncoating. Also similar to reovirus is that the newly synthesized viral mRNAs lack a poly(A) tail at their 3′ end.

Identification of cellular interaction partners of viral proteins is likely to provide a better understanding of the dynamics of RNA replication, virus-mediated cellular modulation and host-range restriction. Recently, we analysed the inter-molecular interactions between the IBDV proteins using the yeast two-hybrid

system [49, 51]. In the present study, we aimed at identifying potential cellular partners for the putative viral RdRp and genome-linked protein, VP1. To this end we applied the inducible LexA-dependent yeast two-hybrid system (Clontech) to screen a cDNA library from bursae of 3-weeks-old chickens using VP1 as a bait. For this, the VP1 gene was subcloned into the yeast expression vector $pLexA_{BD}$ as described previously [49]. The generated $pLexA_{BD}$–VP1 fusion plasmid was introduced into EGY48 yeast cells (Clontech) carrying two reporter genes, i.e. *LEU2* integrated in the genome, and *LacZ* located on the p8op-LacZ reporter plasmid (Clontech). Expression of the VP1-fusion protein was verified by Western blot analysis (data not shown). Activation and repression assays [19] confirmed that the fusion protein by itself did not activate the reporter genes and that it was localized to the nucleus (data not shown).

A yeast two-hybrid cDNA library in $pB42_{AD}$, constructed from poly(A)+ RNA from bursae of 3-weeks-old chickens [5], was screened with $pLexA_{BD}$–VP1 used as a bait. All yeast two-hybrid media, buffers, and protocols were as described in the Clontech Manual for the Matchmaker LexA two-hybrid system and in the Clontech Yeast Protocols Handbook. Plasmids from positive clones were isolated and the inserts were sequenced in both directions. The identity of the respective genes was determined by BLAST analysis. Of the eleven putative VP1 binding proteins that were found more than twice, three were listed by the "Interaction Trap Table of False Positives" (http://www.fccc.edu/research/labs/golemis/main_false.html) as notoriously non-specific interactors, and five were classified as proteins of unknown function, i.e. having no known functional or structural homologues. Other interacting proteins were RAN binding protein 1 (RanBP1) and the putative cyclin G1 interacting protein [accession no: NP_006340]. However, the clone that was frequently recovered (eight isolates) and, in addition, exhibited a more robust β-galactosidase activity, corresponded to part of the translational eukaryotic initiation factor 4AII (eIF4AII) and would thus be the chicken representative of this initiation factor. For this reason we focused our attention on the study of the interaction between VP1 and eIF4AII. An additional consideration was the recent demonstration of another interaction of a viral RdRp, the NS5B protein of hepatitis C virus, with (human) eIF4AII [24].

The eight recovered eIF4AII-homologous cDNAs ranged in size from 983 to 1028 nt. They all mapped to the 3′ end of the eIF4AII gene sequence, five clones encoding 107 aa, one clone encoding 109 aa and two clones encoding 122 aa of the eIF4AII carboxy-terminus (Fig. 1; indicated by asterisks). All cDNAs had the same 662 nt-3′ UTR in which two potential polyadenylation signals occur. The eight cDNAs were derived from at least two different mRNA populations since a comparison of their sequences showed the occurrence of two nucleotide differences in the coding region neither of which, however, affected the predicted amino acid sequence of eIF4AII. As the actual N-terminus of the chicken eIF4AII was unknown we isolated and sequenced the full-length cDNA clone of this protein from our bursae library. A 1845 nt cDNA sequence was obtained for the chicken eIF4AII gene (c-eIF4AII; Gen-Bank accession number AF515726). Its coding sequence of 1221 nt predicts a 407 aa protein. The 3′ terminal part of the gene corresponding to the aa sequence that was common to all eight VP1-interacting

```
c-eIF4AII  1    MSGGSADYSRDHGGPEGMEPDGVIESNWNEIVDNFDDMNLKESLLRGIYA
m-eIF4AII  1    --------N-E-------D------------------------------
m-eIF4AI   1    --ASQDSR---N•--D----E----------S------S---------

c-eIF4AII  51   YGFEKPSAIQQRAIIPCIKGYDVIAQAQSGTGKTATFAISILQQLEIDLK
m-eIF4AII  51   ------------------------------------------------EF-
m-eIF4AI   50   --------------L----------------------------I-----

c-eIF4AII  101  ETQALVLAPTRELAQQIQKVILALGDYMGATCHACIGGTNVRNEMQKLQA
m-eIF4AII  101  --------------------------------------------------
m-eIF4AI   100  A-------------------VM--------S-----------A-V----M

c-eIF4AII  151  EAPHIVVGTPGRVFDMLNRRYLSPKWIKMFVLDEADEMLSRGFKDQIYEI
m-eIF4AII  151  --------------------------------------------------
m-eIF4AI   150  -----I-------------------Y----------------------D-

c-eIF4AII  201  FQKLSTNIQVVLLSATMPMDVLEVTKKFMREPIRILVKKEELTLEGIKQF
m-eIF4AII  201  ------S----------T----------D------------------
m-eIF4AI   200  -----S-T----------S----------D------------------
                                                 122        109
                                                 ★          ★
c-eIF4AII  251  YINVEREEWKLDTLCDLYETLTITQAVIFLNTRRKVDWLTEKMHARDFTV
m-eIF4AII  251  --------------------------------------------------
m-eIF4AI   250  --------------------------------------------------
                107
                ★
c-eIF4AII  301  SALHGDMDQKERDVIMREFRSGSSRVLITTDLLARGIDVQQVSLVINYDL
m-eIF4AII  301  --------------------------------------------------
m-eIF4AI   300  --M-----------------------------------------------

c-eIF4AII  351  PTNRENYIHRIGRGGRFGRKGVAINFVTEEDKRILRDIETFYNTTVEEMP
m-eIF4AII  351  --------------------------------------------------
m-eIF4AI   350  ------------------------M-------T----------SI----

c-eIF4AII  401  MNVALDI
m-eIF4AII  401  -------
m-eIF4AI   400  L------
```

**Fig. 1.** Comparison of the amino acid sequence for chicken eIF4AII (c-eIF4AII) predicted from full-length cDNA, mouse eIF4AII (m-eIF4AII) and mouse eIF4AI (m-eIF4AI). Amino acid sequences were aligned using MSA Version 2.1 provided on the World Wide Web server. Dashes indicate amino acids identical to that of the chicken eIF4AII. The dot indicates a gap inserted by the alignment program. The asterisks mark the start of the the amino acid sequences of the VP1-interacting bursae library clones pB42$_{AD}$-eIF4AII(C-122 aa), pB42$_{AD}$-eIF4AII (C-109 aa) and pB42$_{AD}$-eIF4AII(C-107 aa). Bold letters indicate the start of the carboxy-terminal domain of the "dumbbell" structure of eIF4A according to the published crystal structure of yeast eIF4A [8]. Conserved motifs of eIF4A are boxed. The accession numbers are as follows: c-eIF4AII (AF515726), m-eIF4AII (S00985) and m-eIF4AI (S00986)

eIF4AII-cDNAs showed complete sequence identity. Some differences were, however, observed in its 612 nt-3′ UTR, which lacked a stretch of 53 nucleotides, showed one transition (T to C), and contained an additional three nucleotides (CTT) at the extreme 3′ end (Fig. 2). The deduced amino acid sequence of chicken eIF4AII was compared to that of eIF4AII and eIF4AI from mouse (Fig. 1). The

**3′-UTR**

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eIF4AII(full-length)    1       TAATCCCTGGAGAGGAGATGGTTTGAATGCAGTGCTCGCTGTTGCTGAATAGGCGATTCA
eIF4AII(C-122aa)        1       ------------------------------------------------------------

eIF4AII(full-length)    61      CTTGCATTGTGCTTCTTTCTTTGGGGATATATTGAATCTTGTCTCAATGCTCATAACGGA
eIF4AII(C-122aa)        61      ------------------------------------------------------------

eIF4AII(full-length)    121     TCAGAAATACAGATTTTTGATAAGCGAAGCGACTTTTTGTCGTGAGCTCTTGTGGGGAAA
eIF4AII(C-122aa)        121     ------------------------------------------------------------

eIF4AII(full-length)    181     GCCATTGGCTTTATCCACTTTAGGGTTAGA****************************
eIF4AII(C-122aa)        181     -----------------------------CTGTTGGGGTTGGGTGGAAAGTCATGGGGT

eIF4AII(full-length)    211     **********************AATTTATTTCCTAGTTCCATAGAAGTGGTTGTATTAG
eIF4AII(C-122aa)        241     CTGTAAATTTTTTCTTTATTAGA------------------------------------

eIF4AII(full-length)    248     ATGTTCTTTATCATTTAATAATTTACTTATGGACTAAAAGATATAAGTGCTGTATAAAAT
eIF4AII(C-122aa)        301     ------------------------------------------------------------

eIF4AII(full-length)    308     CAGCCAATTATGTTAAACTAGCCTACCTTCCTTTATTGTATGTACTTACCCTTCAGATTG
eIF4AII(C-122aa)        361     ------------------------------------------------------------

eIF4AII(full-length)    368     AATTGGAAAGGCCTTTCAAAATCTCAAAACTTTTATAAGAGCATTAAAATGCATTTTCGT
eIF4AII(C-122aa)        421     ------------------------------------------------------T--

eIF4AII(full-length)    428     TTGATATGTATTTATTCAATAAAGTATTTAATTAGTGGTAAGTGTGATCTGGACCCTGTT
eIF4AII(C-122aa)        481     ------------------------------------------------------------

eIF4AII(full-length)    488     GCTAAGCCCCAGCAAGCAAGCAATCATACTATTGTCTTAGTTAGGGTTAAACCCAGTAAA
eIF4AII(C-122aa)        541     ------------------------------------------------------------

eIF4AII(full-length)    548     ATTTGCCATATTGCACATGTCTTAATGAAGTTTGAATGTTCAATAAAATACTTCTATATT
eIF4AII(C-122aa)        601     ------------------------------------------------------------

eIF4AII(full-length)    608     CACTTAAA
eIF4AII(C-122aa)        661     --***AAA
                                     Δ
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**Fig. 2.** Sequence alignment of the 3′ UTRs of chicken bursa eIF4AII full-length cDNA and the VP1-interacting chicken bursa eIF4AII(C-122 aa) cDNA. The stop codon is given in bold letters. Dashes indicate identical residues and asterisks mark missing nucleotides. The polyadenylation signals are underlined and the position of poly(A) addition for both cDNAs is indicated by an open triangle beneath the sequence

comparison indicates that the chicken protein is more similar to mouse eIF4AII (98% identity) than to mouse eIF4AI (91% identity). Both isoforms, eIF4AI and eIF4AII, are involved in translation initiation and are functionally interchangeable [52]. More recently, a third member of the eIF4A family, eIF4AIII, has been identified but this protein is more distantly related and its involvement in translation initiation has been questioned in view of its predominant nuclear occurrence [21, 28].

In order to check the specificity of our observed VP1-eIF4AII interaction, the VP1-interacting eIF4AII library clones were retransformed back to yeast either alone or in combination with VP1, with the binding domain vector alone, or with different proteins unrelated to VP1:pVP2 and VP3 of IBDV, and Lamin C (Clontech). In addition, full-length eIF4AII, termed c-eIF4AII(full-length), was

assayed in the yeast two-hybrid system in the same way as the eIF4AII library clones, termed c-eIF4AII(C-107 aa), c-eIF4AII(C-109 aa) and c-eIF4AII(C-122 aa). All transformants failed to induce the reporter genes except for the

**Table 1.** Interactions between IBDV VP1 and different lengths of chicken bursa-specific eIF4AII (ceIF4AII), mouse eIF4AII (meIF4AII) and mouse eIF4AI (meIF4AI) in the Yeast Two-Hybrid System

| $LexA_{BD}$ fusion | $B42_{AD}$ fusion | LEU[a] | LacZ[b] |
|---|---|---|---|
| VP1 | c-eIF4AII(full-length) | − | − |
| VP1 | c-eIF4AII(C-107 aa) | +++ | ++ |
| VP1 | c-eIF4AII(C-109 aa) | +++ | ++ |
| VP1 | c-eIF4AII(C-122 aa) | +++ | ++ |
| VP1 | c-eIF4AII(C-domain[c]) | + | + |
| VP1 | c-eIF4AII(N-domain[d]) | − | − |
| c-eIF4AII(full-length) | VP1 | − | − |
| c-eIF4AII(C-107 aa) | VP1 | + | +/− |
| c-eIF4AII(C-109 aa) | VP1 | + | +/− |
| c-eIF4AII(C-122 aa) | VP1 | + | +/− |
| c-eIF4AII(C-domain) | VP1 | +/− | +/− |
| c-eIF4AII(N-domain) | VP1 | − | − |
| VP1 | m-eIF4AII(full-length) | − | − |
| VP1 | m-eIF4AII(C-107 aa) | +++ | ++ |
| VP1 | m-eIF4AII(C-domain) | + | + |
| VP1 | m-eIF4AII(N-domain) | − | − |
| m-eIF4AII(full-length) | VP1 | − | − |
| m-eIF4AII(C-107 aa) | VP1 | + | +/− |
| m-eIF4AII(C-domain) | VP1 | +/− | +/− |
| m-eIF4AII(N-domain) | VP1 | − | − |
| VP1 | m-eIF4AI(full-length) | − | − |
| VP1 | m-eIF4AI(C-107 aa) | + | + |
| VP1 | m-eIF4AI(C-domain) | +/− | − |
| VP1 | m-eIF4AI(N-domain) | − | − |
| m-eIF4AI (full-length) | VP1 | − | − |
| m-eIF4AI(C-107 aa) | VP1 | − | − |
| m-eIF4AI(C-domain) | VP1 | − | − |
| m-eIF4AI(N-domain) | VP1 | − | − |

[a]Interactions were assayed for leucine autotrophy (LEU): +++; clear growth (strong interaction); +, growth (interaction); +/−, limited growth (weak interaction); −, no growth (no interaction). All controls, including the fusion-proteins by themselves or in combination with Lamin C, or the viral proteins pVP2 and VP3, remained negative

[b]Interactions were assayed for β-galactosidase activity (LacZ): ++, deep-blue colonies (strong interaction); +, blue colonies (interaction); +/−, light-blue colonies (weak interaction); −, white colonies (no interaction). All controls, including the fusion-proteins by themselves or in combination with Lamin C, or the viral proteins pVP2 and VP3, remained white

[c,d]C-domain and N-domain encodes the carboxy- and amino-terminal domain of the "dumbbell" structure of eIF4A [8]

combination of the three eIF4AII library clones with VP1 (Table 1). A similar result, although with a weaker interaction strength, was obtained for the reciprocal combinations of bait and prey fusion-proteins (Table 1). These results demonstrate that eIF4AII(C-107 aa), eIF4AII(C-109 aa) and eIF4AII(C-122 aa) are true positive VP1 interaction partners in the two-hybrid system. They also show that full-length eIF4AII did not interact with VP1 in this assay.

The crystal structure of eIF4A has been reported as a "dumbbell" structure consisting of two compact domains connected by an extended linker [8]. Since the VP1-interacting eIF4AII library clones encode almost the complete carboxy-terminal domain of this "dumbbell" structure (Fig. 1) it was of interest to know whether the interaction is indeed limited to this particular domain. To this end, the carboxy-terminal domain (Fig. 1, c-eIF4AII residues 244–407) and the amino-terminal domain (Fig. 1, c-eIF4AII residues 1–243) of the eIF4AII-"dumbbell" structure were independently assayed for interaction with VP1 in the two-hybrid system. The assay confirmed that the carboxy-terminal domain of eIF4AII, termed eIF4AII(C-domain), but not its amino-terminal domain, termed eIF4AII(N-domain), was able to interact with VP1. It also appeared that the interaction of eIF4AII(C-domain) with VP1 was weaker than that of the shorter C-terminal domains of the eIF4AII library clones (Table 1). The reciprocal combination of the respective bait and prey fusion-proteins gave the same result, although again, as was observed with the eIF4AII library clones, the interaction strength of this combination was weaker.

We also investigated whether the interaction of eIF4AII with VP1 is isoform-II specific. To this end the two isoforms eIF4AI and eIF4AII of mouse (m-eIF4AI and m-eIF4AII) had to be used because the existence and hence the sequence of a putative chicken eIF4AI is unknown. Plasmids pTZ18R-4A and pTZ19-4AII [46] encoding the full-length cDNA sequences of mouse eIF4AI and eIF4AII, respectively, were used as template for preparing the m-eIF4AI and m-eIF4AII constructs. Along with these constructs we additionally tested the N- and C-terminal domains of both these mouse-specific eIF4A isoforms, as well as a truncated form encoding 107 aa of the C-terminus resembling the shortest bursa-specific eIF4AII library clone. It is of interest to note that the amino acid sequence of m-eIF4AII(C-107 aa) is 100% identical to that of c-eIF4AII(C-107 aa) (Fig. 1). The results showed that for both mouse-specific eIF4A isoforms I and II the 107 aa polypeptide bound to VP1, although the strength of the interaction with the isoform I polypeptide was weaker. In the reciprocal combination this truncated protein, m-eIF4AI(C-107 aa), even failed to activate *LEU* or *LacZ* reporter activity. Consistently, also the C-terminal domains of both m-eIF4AII and m-eIF4AI were able to interact with VP1, although the isoform I interaction appeared to be very weak as only one of the two reporters was sensitive enough to allow detection of this interaction. Moreover, the C-domain of m-eIF4AI failed to interact with VP1 in its reciprocal combination of bait and prey. Finally, the N-terminal domain as well as the full-length form both of m-eIF4AI and of m-eIF4AII, were unable to interact with VP1. All these results are similar to those found for the chicken-specific eIF4AII(N-domain) and eIF4AII(full-length).

Altogether, these results indicate that the carboxy-terminal domain of both m-eIF4A isoform I and II is able to interact with VP1, but that isoform II has the strongest interaction.

Next we wanted to confirm the specificity of the interaction of eIF4AII(C-terminus) with VP1 in an independent manner. With this aim we co-expressed the VP1 protein with various forms of the eIF4A proteins in avian QM5 cells [2] using a transient fowl pox virus expression system as described previously [4], and analysed association of the proteins by radio-immunoprecipitation (RIP) assay. For this, cDNA of chicken eIF4AII and of mouse eIF4AI and eIF4AII, as well as derivates thereof (i.e. fragments encoding the polypeptides C-107 aa, C-domain and N-domain) were cloned as a fusion protein with a c-myc epitope tag in the transcription plasmid pGBKT7 (Clontech) behind a T7 RNA polymerase promoter. We fused the c-myc tag (N-terminally) to eIF4A because no antibody against eIF4A(C-terminus) was available. For the expression of VP1 we used plasmid pHB34Z, in which the full-length genomic cDNA of segment B of IBDV is cloned between a T7 RNA polymerase promoter and the autocatalytic hepatitis delta virus ribozyme sequence [5]. The eIF4A encoding plasmids were co-transfected with plasmid pHB34Z into QM5 cells that were previously infected with a recombinant fowlpoxvirus expressing T7 RNA polymerase (as described in [50]). Forty-eight hours post-transfection, cells were metabolically labeled for 3 h with [$^{35}$S]methionine, cell lysates were prepared and subjected to immunoprecipitation with either anti-VP1 [49] or anti-c-myc serum (Clontech), as described in [50]. The immunoprecipitates obtained were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and the labeled proteins visualized by autoradiography. Mock-transfected cells and cells transfected with either pHB34Z or the eIF4A-encoding plasmids alone were used as controls for each immunoprecipitation.

Both antibodies were found to precipitate only their cognate protein, indicating that they did not exhibit cross-reactivity (Fig. 3A, lanes 13–15 and Fig. 3B, lanes 6–8; data of eIF4A-derivatives not shown except for c-eIF4AII(C-107 aa)). Using the anti VP1-serum, comparable amounts of VP1 were detected in the different transfected QM5 cells (Fig. 3A, lanes 1–12). Full-length c-eIF4AII, m-eIF4AI and m-eIF4AII each failed to be co-precipitated with VP1 (Fig. 3A, lanes 1, 5 and 9). In contrast, all C-107 aa constructs were co-precipitated with VP1, indicating that these polypeptides were interacting with VP1 (Fig. 3A, lanes 2, 6 and 11). Notably, the binding of m-eIF4AI(C-107 aa) to VP1 (Fig. 3A, lane 6) appeared to be weaker than that of m-eIF4AII(C-107 aa) (Fig. 3A, lane 11). Of the C-domain derivatives, m-eIF4AI failed to be co-precipitated (Fig. 3A, lane 7), whereas both c-eIF4AII and m-eIF4AII were co-precipitated though in much lower quantities than their C-107 aa derivative (Fig. 3A, lane 3 and 10). No co-precipitation with VP1 was observed of any of the N-domain derivatives (Fig. 3A, lanes 4, 8 and 12). Using the anti-c-myc antiserum for the reciprocal immunoprecipitations, all tagged eIF4A-derivatives were clearly precipitated. However, in neither case could any co-precipitating VP1 be detected (Fig. 3B, lanes 1–4; data of the m-eIF4A (-derivatives) not shown). This was equally the case when we tested the derivative c-eIF4AII(C-122 aa) for interaction with VP1 (Fig. 3B, lane 5).

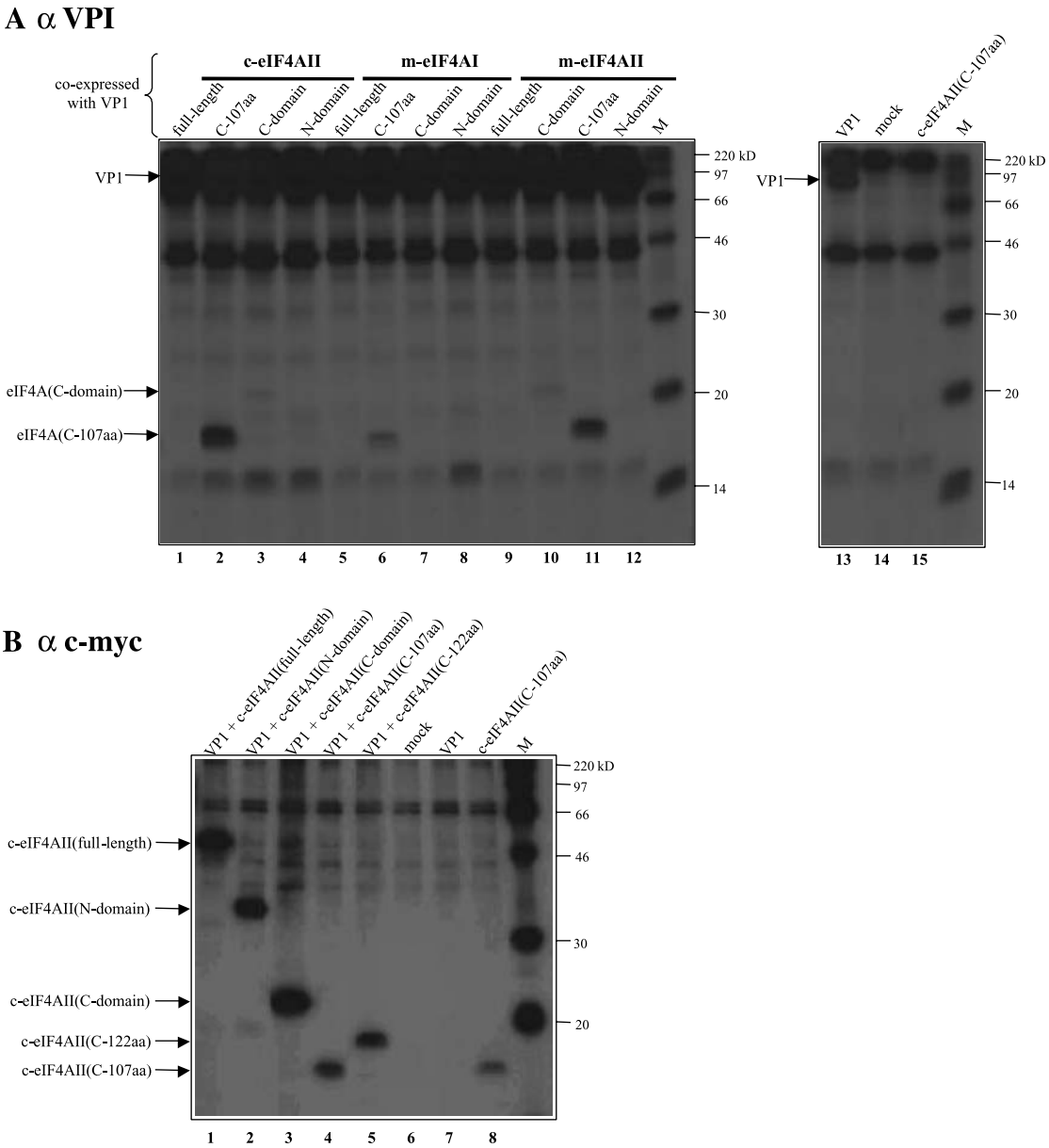


**Fig. 3.** Radio-immunoprecipitation analysis of VP1-eIF4A interaction in transfected QM5 cells. QM5 cells were (co-)transfected with plasmids pHB34Z and/or pGBKT7-eIF4A (derivatives) expressing the indicated polypeptides. At 48 h post transfection cells were metabolically labeled for 3 h with [$^{35}$S]methionine. Subsequently, cells were lysed and immunoprecipitated with anti-VP1 serum (**A**) or with anti-c-myc serum (**B**) followed by SDS-PAGE (18% polyacrylamide) and autoradiography. Mock-transfected cells were used as a control for each immunoprecipitation. Positions of the precipitated polypeptides and sizes of marker proteins (*M*) are indicated

The observation that only the carboxy-terminal domain of eIF4A, not the full-size protein was able to interact with VP1 prompted us to test the idea that in IBDV-infected cells the eIF4A protein might be proteolytically cleaved to generate

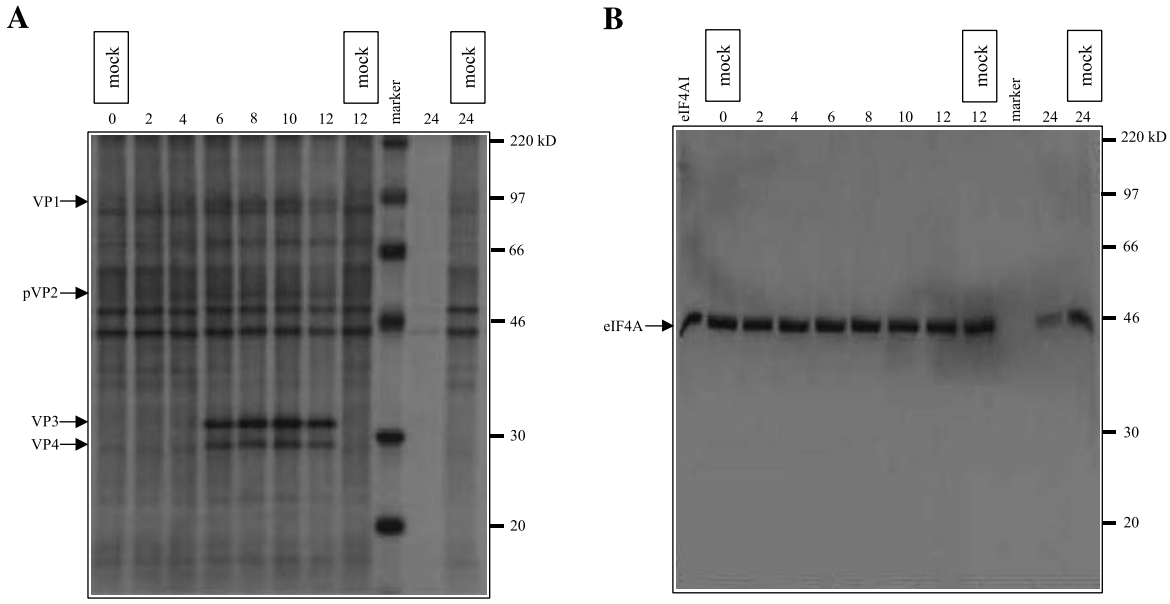


**Fig. 4.** Analysis of host cell protein synthesis within IBDV-infected cells. QM5 cells were infected with IBDV strain CEF94. (**A**) Fifteen min prior to the indicated times (hours post-infection) cells were labeled for 15 min with [$^{35}$S]methionine and subsequently lysed. The cell extracts were analysed by SDS-PAGE (12% polyacrylamide) and autoradiography (**A**). Alternatively, cells infected in parallel with those analysed in **A** were harvested at the same times without metabolic labelling. Cell extracts were prepared and analysed by SDS-PAGE (12% polyacrylamide) and immunoblotting with polyclonal anti-eIF4A serum (**B**). Mock-infected QM5 cell extracts (lanes mock) were analysed in parallel at indicated times. Purified eIF4AI was used as a positive control for the immunoblotting (lane eIF4AI). Positions of viral proteins, eIF4A and molecular size markers are indicated

a carboxy-terminal polypeptide. Such a process would not be without precedent as a similar cleavage has been demonstrated for eIF4AI in foot-and-mouth disease virus (FMDV)-infected cells [29]. Thus, we assessed the effect of IBDV infection on host eIF4A by immunoblotting of cell extracts with a polyclonal antibody specific for eIF4A.

First, host cell protein synthesis and production of virus-encoded proteins were monitored as a function of time during infection (Fig. 4A). Monolayer cultures of 80% confluency of QM5 cells were infected with IBDV strain CEF94 (MOI = 5). After 1 h of virus absorption, QT35-medium (Gibco-BRL) supplemented with 1% foetal calf serum was added to the cells, and incubation was continued until the indicated times (time zero was the time when virus was added to the cells). Where appropriate, 15 min prior to harvesting, cells were washed 3 times with methionine-free EMEM medium (Gibco-BRL) and subsequently labeled for 15 min with 20 μCi/ml of [$^{35}$S]methionine (Amersham) in methionine-free EMEM medium (Gibco-BRL). As controls, mock-infected cells were similarly labelled. Cell extracts were prepared on ice with 1× PBS-TDS lysis buffer [50]. Labeled proteins were subjected to SDS-PAGE (12%) and visualized by

autoradiography. No shut-off of host cell polypeptide synthesis was observed in infected cells indicating that the IBDV infection generally did not interfere with host protein translation. Virus-specific protein synthesis was first detected at 4 h and was maximal between 6 and 12 h postinfection (p.i.). The cells started to show cytopathic effect (CPE) by about 12 h p.i. while at 24 h p.i., when the experiment was terminated, nearly all cells appeared to have succumbed. At this stage almost no protein synthesis was detected.

The effect of IBDV infection on eIF4A was assessed by immunoblotting of the gel followed by probing of the blotted proteins with a polyclonal rabbit antiserum against eIF4A (1:3,000; gift from Dr. C. Kuhlemeier, University of Berne, Switzerland). Thirty ng of purified rabbit eIF4AI [46] was used as a positive control for the immunoblotting. Detection on X-ray film was achieved by using peroxidase-linked goat anti-rabbit immunoglobulins (Ig) (1:3,000; DAKO) and chemiluminescent reagents (Pierce). Full-length eIF4A migrated at about 46 kD as a doublet, most likely representing the eIF4AI and eIF4AII isoforms, which were present in both infected and mock-infected cells (Fig. 4B). It was apparent that the infection with IBDV did not result in a specific cleavage of eIF4A nor in a general proteolytic degradation of the protein as no cleavage products nor a significant loss of full-length eIF4A was detected during this time course under these conditions. The decrease of the overall quantity of full-length eIF4A at 24 h p.i. was attributed to the severe CPE at this stage of infection.

It is becoming remarkably clear that the birnavirus VP1 protein is an impressively multifunctional viral component, exhibiting a range of activities throughout the viral life cycle. Firstly, as the putative RNA-dependent RNA polymerase, VP1 is expected to carry out all those diverse functions required for the replication and transcription of the binary dsRNA genome that occur within viral particles and in the cell's cytoplasm [48]. Secondly, it acts as a primase in the generation of viral mRNAs, as a result becoming part of these mRNAs through covalent linkage at their 5′ end [35]. Furthermore, by its interaction with the inner capsid component VP3 [10, 31, 36, 49, 50] the protein is also considered to be instrumental in the encapsidation of viral RNAs during particle morphogenesis. VP1 also associates with itself, but the significance of this interaction has not yet been established [49]. In the present study we focussed, for the first time, on interacting host cell partners of VP1 by using the yeast two-hybrid system. Our observation that VP1 specifically binds to the C-terminus of eukaryotic translation initiation factor eIF4AII suggests an involvement of this protein in translation initiation. This interaction therefore further extends the list of potential functions of this viral protein.

eIF4A is the prototypic member of the DEAD-box family of RNA helicases [30]. Together with eIF4B it participates in the initiation of polypeptide synthesis by facilitating the melting of RNA secondary structures present in the 5′ UTR of mRNAs required for the efficient recruitment of the 40S ribosomal subunit [reviewed in 18].

An interaction of VP1/VPg with eIF4AII is intriguing particularly because IBDV mRNAs are not capped but most likely contain a VPg at the 5′ end, similar to the genomic RNA [15, 35]. A direct interaction of eIF4A with VPg may be

a key event in the establishment of IBDV infection. It may reveal a novel cap-independent mechanism for the initiation of translation in which the binding of eIF4A to VPg mediates the recruitment of the 40S ribosomal subunit to the 5′ end of the viral mRNA. Thus, while eIF4E is essential for initiation of translation of capped mRNAs, and proteolytically cleaved eIF4G for that of picornaviral uncapped mRNA [25], eIF4A may have a similar function for IBDV mRNA.

For a subset of viral and cellular mRNAs, cap-independent initiation of translation is accomplished by internal binding of the ribosome at or upstream of the initiation codon mediated by an internal ribosomal entry site (IRES) in the 5′ UTR [reviewed in 37]. The possibility of IRES-mediated cap-independent translation has not been investigated for IBDV, nor for other birnaviruses, but is not unlikely to occur. Although the nucleotide sequence of the 5′ UTR of IBDV RNA is relatively short (segment A: 96 nt, segment B: 111 nt) compared to that of many viral IRES-containing mRNAs (about 200–500 nt), both segments have a theoretical potential to form Y-shaped or hairpin stem and loop secondary structures [23, 42]. These structures resemble those proposed to occur in some cellular IRESes [26]. However, there is no convincing evidence supporting the idea that these cellular IRES structures are of significance for translation initiation. More interestingly, it was found that some mRNAs may recruit ribosomes by base pairing with ribosomal RNA [9, 44, 53]. Sequences as short as 9 nt have been shown to possess such IRES activity [9]. These short IRES-modules have complementarity to the 18S rRNA occurring in 40S ribosomal subunits. A similar feature might be the case for IBDV mRNA. A highly conserved sequence of 13 nt in the 5′ UTR of both segments, located exactly 19 nt upstream of the respective start codons for the nearest ORF (VP5 gene in segment A, VP1 gene in segment B), has complementarity to the chicken reticulocyte 18S rRNA and could thus function as a binding site for the 40S ribosomal subunit [42]. A tenable hypothesis is that an interaction of the C-terminus of eIF4A with VPg might be required to open mRNA secondary structure to allow ribosome binding.

The eIF4AII encoding cDNA clones that were identified in the yeast two-hybrid screen all corresponded to the carboxy-terminal domain of the protein. The specific interaction of VP1 with this particular eIF4AII domain was confirmed in various experiments, not only in the two-hybrid system but also in the co-immunoprecipitation assays. No association of VP1 was observed with full-length eIF4AII, also not in IBDV-infected cells (data not shown) by different co-immunoprecipitation assays. We then investigated whether in infected cells an eIF4AII carboxy-terminal fragment is generated by some kind of cleavage, similar to what has been found for eIF4AI in FMDV-infected cells [29]. No breakdown of eIF4AII was, however, observed, the protein appearing as stable as in non-infected cells. Our interpretation of these results is that the formation of VPg-eIF4AII complexes might be conformation dependent and transient. In its free form the initiation factor does not expose its VP1 binding site. Only when engaging in the formation of a translation initiation complex this site becomes available as a result of conformational changes induced in the eIF4AII structure

by its interaction with other partners involved in the assembly of the complex such as eIF4G or 3′ end mRNA elements. In fact, various studies suggest that eIF4A does indeed adopt different conformations. (i) Mammalian eIF4A undergoes a cycle of ligand-dependent conformational changes as it binds its substrates, hydrolyzes ATP, and releases products [32, 33]. (ii) In its crystallized form, eIF4A has a 'dumbbell' structure in which the amino- and carboxy-terminal domains occur as separately folded entities connected by an extended, 11-residue linker [8]. In solution, the linker between the domains is relatively flexible, allowing eIF4A to adopt many different conformations [8]. (iii) eIF4A is expected to interact only transiently with eIF4G, cycling in and out of the eIF4F complex, thereby changing its conformation [45]. Altogether, these considerations support our interpretation, although we can of course not exclude that full-length eIF4A has an overall weak affinity for VP1.

The immunoprecipitation analyses demonstrated that co-expressed VP1 and c-myc-tagged carboxy-terminal eIF4AII polypeptides form stable complexes, particularly VP1/C-107 aa complexes, that are efficiently immunoprecipitated with VP1 antibodies (Fig. 3A). Somewhat surprisingly, these complexes were not precipitable in the reciprocal assay using c-myc antibodies (Fig. 3B). The c-myc antibodies clearly recognized the tagged c-eIF4AII proteins but no VP1 was co-precipitated. It seems feasible that the binding of antibody to the tag induces a conformational change in the eIF4AII polypeptide that destabilizes its complex with VP1. The tag was fused to the amino-terminus of the c-eIF4AII polypeptides. We have not tested whether attachment to the carboxy-terminus of the polypeptides would have made a difference.

It is quite remarkable that our two-hybrid screening only yielded eIF4A-derivatives of isoform type-II as VP1-interacting proteins and that no clones of the functionally equivalent isoform type-I were isolated. eIF4AII strongly resembles eIF4AI (in mice the two isoforms have 91% identity) though its tissue-specific expression [43] and its developmental regulation [38] differ from those of eIF4AI. While the carboxy-terminal polypeptides (C-107 aa) of both the mouse-specific eIF4AI and eIF4AII isoforms were able to interact with VP1, the strength of the interaction with isoform type-I was consistently weaker. This suggests that VP1 preferentially associates with eIF4AII. Data indicate that this isoform is also held more strongly in the eIF4F complex than is eIF4AI [12].

The interaction of VP1 with the carboxy-terminal domain of eIF4AII reported here suggests yet another viral strategy on the protein translation battlefield. Future studies will be required to confirm and establish the functional significance of this interaction for viral multiplication, particularly whether VP1, when linked to viral RNA (i.e. as VPg) can indeed bind the initiation factor and support translation initiation of viral RNA, without compromising host mRNA translation. Another interesting question relates to the possible occurrence of 5′-3′ interactions in the IBDV mRNAs and to the binding partners that mediate these interactions. These studies will undoubtedly reveal additional striking features of the biology of these fascinating viruses.

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