RESEARCH ARTICLE

The RISC component VIG is a target for dsRNA-independent protein kinase activity in *Drosophila* S2 cells

Konstantin I Ivanov[1,*], Timofey V Tselykh[2,4], Tapio I Heino[2,3] and Kristiina Mäkinen[1,*]

[1]Department of Applied Biology, [2]Institute of Biotechnology, Developmental Biology Program and [3]Department of Biological and Environmental Sciences, University of Helsinki, FIN-00014, Finland. [4]Present address: Molecular and Cancer Biology Research Program, Institute of Biomedicine, Biomedicum Helsinki, University of Helsinki, FIN-00014, Finland

Correspondence to: Konstantin I Ivanov or Kristiina Mäkinen, *Email*: Konstantin.Ivanov@helsinki.fi; Kristiina.Makinen@helsinki.fi, *Tel*: +358 9 191 58349 (Konstantin Ivanov); +358 9 191 58342 (Kristiina Mäkinen), *Fax*: +358 9 191 58633

Journal of RNAi and Gene Silencing (2005), 1(1), 12-20

(Received 16 May 2005; Revised 06 July 2005; Accepted 07 July 2005, Available online 27 July 2005; Published 12 August 2005)

ABSTRACT

RNA interference (RNAi) is mediated by a multicomponent RNA-induced silencing complex (RISC). Here we examine the phosphorylation state of three *Drosophila* RISC-associated proteins, VIG, R2D2 and a truncated form of Argonaute2 devoid of the nonconserved N-terminal glutamine-rich domain. We show that of the three studied proteins, only VIG is phosphorylated in cultured *Drosophila* cells. We also demonstrate that the phosphorylation state of VIG remains unchanged after cell transfection with exogenous dsRNA. A sequence similarity search revealed that VIG shares significant similarity with the human phosphoprotein Ki-1/57, a known *in vivo* substrate for protein kinase C (PKC). *In vitro* kinase assays followed by tryptic phosphopeptide mapping showed that PKC could efficiently phosphorylate VIG on multiple sites, suggesting PKC as a candidate kinase for VIG phosphorylation *in vivo*. Taken together, our results identify the RISC component VIG as a novel kinase substrate in cultured *Drosophila* cells and suggest a possible involvement of PKC in its phosphorylation.

KEYWORDS: *RNA interference, RNAi, RISC, Vasa Intronic Gene, VIG, Argonaute, R2D2*

INTRODUCTION

In *Drosophila,* one of the most versatile and thoroughly studied model organisms, several proteins have been implicated in RNAi induced by exogenous dsRNA. Dicer 2 (DCR-2), an RNAse III family nuclease, cleaves the introduced long dsRNA into multiple short interfering RNAs (siRNAs) instructing a multicomponent RNA-induced silencing complex (RISC) to destroy homologous target mRNAs (Zamore et al, 2000; Bernstein et al, 2001; Lee et al, 2004). A protein named R2D2 stably associates with DCR-2 (Liu et al, 2003) and functions as a sensor for siRNA asymmetry (Tomari et al, 2004). The core RISC protein Argonaute2 (Ago2) (Hammond et al, 2001) is the catalytic engine behind target mRNA cleavage (Liu et al, 2004; Song et al., 2004). Other RISC components include Tudor-SN (TSN) (Caudy et al, 2003), dFMR1 (alternatively named dFXR; Caudy et al, 2002; Ishizuka et al, 2002) and a protein named VIG, encoded from within an intron of the Vasa gene (Caudy et al, 2002). Four more proteins, Armitage, Spindle E, Rm62, and Dmp68 are thought to be involved in the assembly of RISC (reviewed by Meister and Tuschl, 2004; Sontheimer, 2005).

Reversible protein phosphorylation is a central mechanism controlling protein function in living cells. At least one protein component of the *Drosophila* RISC, dFMR1, is phosphorylated *in vitro* and *in vivo* (Siomi et al, 2002). Protein kinase CK2 (formerly known as casein kinase 2) has been implicated in the phosphorylation of dFMR1. The phosphorylation site has been mapped to Ser406, which is highly conserved among FMR family members from several species. Human and murine orthologs of dFMR1 are primarily phosphorylated on the same con-

served serine residue within the CK2 consensus sequence (Siomi et al, 2002; Ceman et al, 2003). Phosphorylation at this site regulates dFMR1 oligomerization and RNA binding, suggesting that the biological functions of dFMR are regulated by phoshorylation (Siomi et al, 2002). Phosphorylation analysis of other proteins involved in RNAi has not been reported. The aim of this study was to examine the phosphorylation status of VIG, R2D2 and Ago2 in cultured *Drosophila* cells.

MATERIALS AND METHODS

Construction of expression plasmids and stable transfection

The cDNA clones RE04347 (Ago2, CG7439), LD07162 (VIG, CG4170) and AT28705 (R2D2, CG7138) were obtained from the MRC Geneservice, Cambridge, UK. The coding sequences of VIG, R2D2 and Ago2 were PCR-amplified from the corresponding cDNA clones using the primers shown in Table 1. The PCR products were topoisomerase-cloned into pMT/V5-His-TOPO expression vector (Invitrogen, Carlsbad, CA) containing the metal-inducible metallothionein promoter and the C-terminal V5-polyhistidine (His) tag. Stable cell lines were generated according to the *Drosophila* Expression System protocol (Invitrogen). Recombinant protein expression was assayed by western blotting with anti-V5 antibodies.

Table 1. Oligonucleotide primers used in the present study

Purpose	Polarity	Sequence 5' → 3'
R2D2 amplification	sense	ACC ATG GAT AAC AAG TCA GCC GTA TCT GCT
	antisense	AAT CAA CAT GGT GCG AAA ATA GTC TAT
VIG amplification	sense	ACC ATG GAC AGC GCC GGT AAA AAT CGT TAT
	antisense	AGC CAG AGT GGG AAA CTG ACG CTC ATC
Ago2a amplification	sense	ACC ATG GCA CCT TCT GTG GCA TAC CAC TAT
	antisense	GAC AAA GTA CAT GGG GTT TTT CTT CAT
Actn dsRNA preparation	sense	TAA TAC GAC TCA CTA TAG GGA TCG AGC GGC AAA TGG ATG CTG
	antisense	TAA TAC GAC TCA CTA TAG GGC TTG TCG GCT GCC AGG ATT CGG

Note: The sequence corresponding to the T7 RNA polymerase binding site is underlined.

Enzymatic preparation of dsRNA

A 585 nt-long fragment of the largest exon of the α-actinin gene was PCR-amplified from a *Drosophila* cDNA library. Each of the PCR primers incorporated a 5' T7 RNA polymerase minimum binding site (see Table 1 for primer sequences). *In vitro* transcription, annealing and purification of dsRNA were carried out using a MEGAscript RNAi kit according to the manufacturer's protocol (Ambion, Austin, TX).

RNAi experiments

S2 cells were resuspended at a final concentration 10^6 cells/ml in serum free Schneider's medium. Viability was examined by trypan blue dye exclusion. dsRNA was added to the medium at a concentration of 15-30 µg per 10^6 cells. Cells were mixed with dsRNA by gentle swirling and incubated for 1 hr at room temperature. Subsequently, Schneider's medium supplemented with 10% (v/v) fetal calf serum was added and the cells were incubated for an additional 5 days to allow for turnover of the target protein. The efficiency of silencing was determined by immunoblotting of cell lysates with a specific antibody.

Metal affinity precipitation and selective phosphoprotein staining

5-10 x 10^6 stably transfected cells, treated or untreated with α-actinin dsRNA, were incubated for 18 hr in complete Schneider's medium containing 500 µM $CuSO_4$. Cells were collected by centrifugation, rinsed with PBS, and immediately lysed in 4 ml of buffer A (6 M guanidine hydrochloride, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0). The lysates were cleared by centrifugation at 16000 x g for 10 min and incubated on a shaker for 30 min with 100 µl of Ni-NTA agarose (50% slurry pre-equilibrated in buffer A; Qiagen, Valencia, CA). The resin was collected by centrifugation at 400 x g for 5 min and washed with 10 ml of buffer A. Supernatant was carefully removed and washing was repeated with buffer B (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, pH 8.0), then with buffer C (same composition as buffer B, pH 6.0). His-tagged proteins were eluted with 100 µl of buffer E (same composition as buffer B, pH 4.5) and subjected to SDS-PAGE. Gels were stained with ProQ-Diamond phosphoprotein stain (Molecular Probes, Eugene, OR) and SYPRO Ruby total protein stain (Molecular Probes) according to the manufacturer's protocol. Gel images were acquired with a Fuji FLA-5100 system using a 532 nm laser and a 575 nm long pass filter (Fujifilm, Tokyo, Japan).

Metabolic [^{32}P]-orthophosphate labelling and immunoprecipatation

5-10 x 10^6 stably transfected S2 cells were rinsed once and incubated overnight in phosphate/yeastolate-depleted Schneider's medium (Specialty Media, Phillipsburg, NJ) containing 250 µCi/ml [^{32}P]-orthophosphate (HCl-free; Amersham Pharmacia Biotech, Uppsala, Sweden) and 500 µM $CuSO_4$. Prior to labeling, cells were either treated or untreated with α-actinin dsRNA. Labeled cells were collected by centrifugation (250 x g, 10 min), solubilized in NET buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 25 mM NaF, 1 mM Na_3VO_4, 0.5% Nonidet P-40) containing 1% SDS and immediately boiled for 15 min. The lysates were transferred to fresh tubes and diluted (1:10) with NET buffer supplemented with 2% (w/v) BSA and Complete EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland). Presoaked protein A-Sepharose (Amersham Pharmacia Biotech) was added at 1% (w/v) to the diluted lysate and incubated on a rotator for 1.5 hr at 6°C to remove Sepharose-binding proteins. Following centrifugation 1500 x g for 10 min, rabbit polyclonal anti-V5 antibody (Sigma, St. Louis, MO) prebound to protein A-Sepharose was added to the supernatant and incubated on a rotator overnight at 6°C. The immunoprecipitate was collected by centrifugation at 1000 x g for 10 min, washed four times with NET buffer, and analyzed by western blotting with mouse anti-V5 IgG (Invitrogen). The membrane was exposed to an im-

age plate for 48 hr and then scanned using a phosphor imager (Fujifilm).

***In vitro* phosphorylation assays**

VIG-expressing cells were immunoprecipitated with rabbit anti-V5 antibody as described above. Precipitated immunocomplexes were washed with kinase buffer (20 mM HEPES, pH 7.4, 1.7 mM $CaCl_2$, 5 mM $MgCl_2$, 1 mM dithiothreitol) and incubated for 25 min at 30°C in the same buffer containing 0.6 mg/ml phosphatidylserine, 2 μM ATP, 1 μCi [γ-^{32}P]ATP and 25 ng of purified rat brain PKC (a mixture of predominantly α, β and γ isoforms; Promega, Madison, WI). The reactions were supplemented with 2 mM $MnCl_2$ and further incubated for 15 min at 30°C in the absense or presence of 7 U/μl recombinant lambda protein phosphatase (New England Biolabs, Beverly, MA). The immunoprecipitates were washed three more times with NET buffer to remove unincorporated [γ-^{32}P]ATP and analyzed by western blotting and autoradiography as described in the previous paragraph.

Enzymatic dephosphorylation assays

Metal affinity-precipitated proteins were microdialyzed against milli-Q water to remove urea and other salts. Dialyzed proteins were incubated for 30 min at 30°C in a reaction buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM Na_2EDTA, 5 mM dithiothreitol, 0.01% Brij 35) supplemented with 2 mM $MnCl_2$ in the presence or absence of 7 u/μl recombinant lambda protein phosphatase (New England Biolabs). Samples were resolved by SDS-PAGE and gels were stained with Pro-Q Diamond and SYPRO Ruby. Alternatively, gels were immunoblotted with anti-V5 antibodies.

Two-dimensional tryptic phosphopeptide mapping

Tryptic phosphopeptide mapping was carried out as described previously (Ivanov et al, 2003).

RESULTS

Analysis of VIG, R2D2 and Ago2 expression in stable cell lines

Drosophila Schneider 2 (S2) cells have proven to be a powerful tool for analysis of protein function and dissection of biochemical pathways. In this study, we generated stable S2 cell lines to examine the phosphorylation status of VIG, R2D2 and Ago2. Western blotting with anti-V5 antibodies confirmed that the cell lines expressed R2D2 and VIG (Figure 1, left panel). The size of each protein corresponded to the predicted molecular weight of the fusion protein containing the V5 and His epitope tags. No protein reactive with anti-V5 antibodies was detected in lysates of nontransfected cells (Figure 1, lanes WT).

Both R2D2 and VIG were expressed at levels high enough to allow metal affinity purification and subsequent phosphorylation analysis. However, cells stably transfected with a plasmid carrying the complete open reading frame of the Ago2 gene showed very low expression, often below the detection limit of western blotting. To overcome this problem, we followed the approach of Hammond et al (2001) and deleted the N-terminal domain of Ago2 composed of glutamine-rich repeats. The deletion resulted in a shorter protein that initiates from the second methionine codon of the open reading frame, retaining the functionally important PAZ and PIWI domains (Cerutti et al, 2000; Carmell et al, 2002). The stable cell line expressing the truncated form of Ago2 (designated further as Ago2a) produced significantly more recombinant protein (Figure 1, right panel) than the one expressing the full-length Ago2.

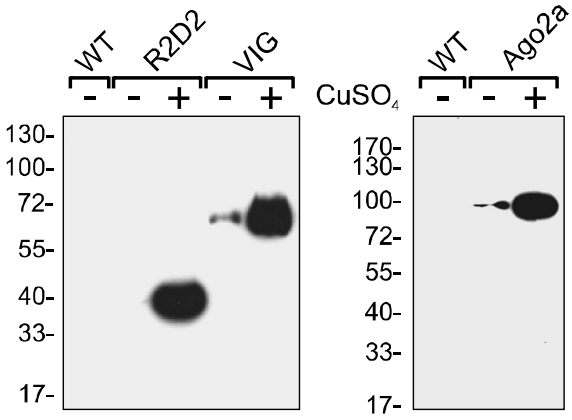

Figure 1. Expression of recombinant R2D2, VIG and Ago2a in cultured S2 cells. Stable cell lines were either treated (+) or untreated (-) with $CuSO_4$ to modulate expression of the C-terminal V5-His fusion proteins. Shown are immunoblots of total cell lysates probed with anti-V5 antibodies. The first lane in each blot contained the lysate of wild type (WT) S2 cells. Molecular mass standards (in kilodaltons) are shown at left on each blot.

VIG, but not R2D2 or Ago2a, is phosphorylated *in vivo*

Since R2D2 and VIG fusion proteins were expressed in stable cell lines at relatively high levels, we examined their *in vivo* phosphorylation status by selective phosphoprotein staining. The proteins were isolated by metal affinity precipitation, resolved by gel electrophoresis and stained with Pro-Q Diamond fluorescent stain (Figure 2A, left panel) that selectively detects phosphoproteins (Steinberg et al, 2003). Although the most intense bands in the Pro-Q Diamond-stained gels usually correspond to phosphoproteins, nonspecific background staining may also be observed with high levels of sample. To distinguish between a low-abundance phosphoprotein and a high-abundance non phosphorylated protein, we stained the same gels for total protein with a quantitative SYPRO Ruby stain (Figure 2A, right panel). The relative intensities of total protein bands were compared with those observed in the Pro-Q Diamond-stained gels. Comparison of the staining patterns revealed that VIG was specifically stained with Pro-Q Diamond, suggesting that it is a phosphoprotein (Figure 2A, lane 4). In contrast, only background staining was observed for R2D2 (Figure 2A, lane 2).

Because the expression levels of Ago2a were lower than those obtained for R2D2 and VIG, we employed two independent experimental approaches to test whether Ago2a is a phosphoprotein. The first approach, based on gel staining with Pro-Q Diamond, showed no phosphorylation of Ago2a (Figure 2B, lane 2). To verify this result, we took a

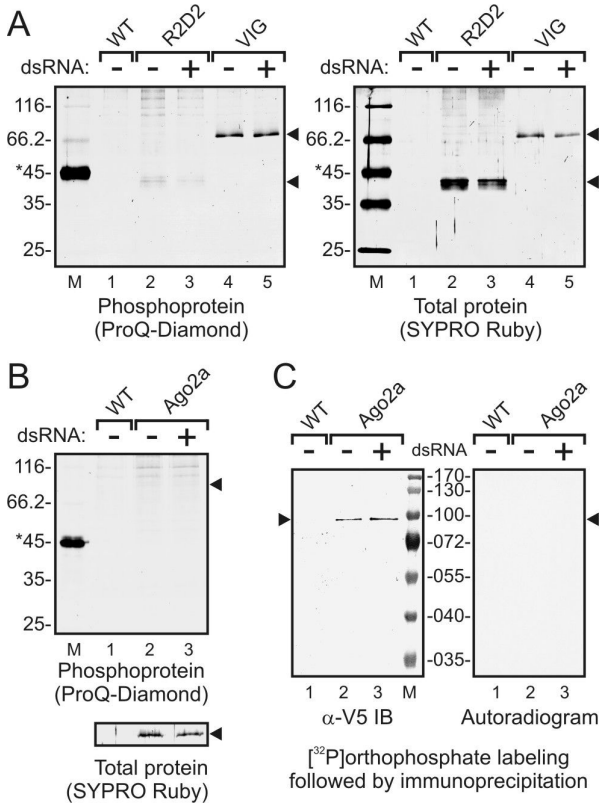

Figure 2. Phosphorylation status of R2D2, VIG and Ago2a in cultured S2 cells. Stable cell lines were incubated in the absence (-) or presence (+) of *Actn* dsRNA, and expression of R2D2, VIG (A) or Ago2a (B) was induced with $CuSO_4$. The proteins were isolated by metal affinity precipitation, resolved by SDS-PAGE, and gels were stained with ProQ-Diamond and SYPRO Ruby. The first lane in each gel contained molecular mass marker proteins including one phosphoprotein (ovalbumin) that served as a positive control for ProQ-Diamond staining (marked with an asterisk). (C) Stable cell line expressing Ago2a was incubated in the absence (-) or presence (+) of *Actn* dsRNA and metabolically labeled with [^{32}P]-orthophosphate. The immunoprecipitated Ago2a was analyzed by western blotting with anti-V5 IgG (left panel) and the membrane was subjected to autoradiography (right panel). The sizes of marker proteins are indicated in kilodaltons, and the positions of R2D2, VIG and Ago2a are marked with arrowheads. Material precipitated from wild type (WT) S2 cells was used as a negative control.

second approach based on radioactive labeling and immunoprecipitation. Stably transfected cells expressing Ago2a were metabolically labeled with [^{32}P] orthophosphate and the protein was immunoprecipitated with anti-V5 antibody. In agreement with the result obtained with Pro-Q Diamond staining, the immunoprecipitated Ago2a did not detectably incorporate [^{32}P] orthophosphate (Figure 2C, lane 2). Thus, we concluded that among the studied proteins, VIG was the only protein phosphorylated in S2 cells.

To further verify that VIG is a phosphoprotein, we performed *in vitro* dephosphorylation assays with lambda protein phosphatase (λ-PPase). VIG was isolated from induced cells by metal affinity precipitation, treated with λ-PPase and subjected to gel electrophoresis followed by Pro-Q Diamond staining. The staining was substantially reduced by phosphatase treatment (Figure 3) unequivocally confirming that VIG was modified by phosphorylation.

The phosphorylation status of VIG, R2D2 and Ago2a remains unchanged after cell treatment with dsRNA

S2 cells can take up long dsRNA directly from the culture medium (Clemens et al, 2000). This makes these cells ideal for studying protein phosphorylation events induced by exogenous dsRNA. When introduced into the cells, dsRNA facilitates sequence-specific degradation of cognate mRNA through RNAi, leading to selective depletion of the encoded protein. In this study, we incubated cells with dsRNA corresponding to a fragment of the *Drosophila* α-actinin (*Actn*) gene encoding an actin filament-binding protein (Otey and Carpen, 2004). After dsRNA treatment, cultures were grown for five more days, the maximum time normally allowed for protein depletion. During all this time the cells were viable and maintained normal morphology. The RNAi efficiency was estimated by comparing α-actinin levels in dsRNA-treated and untreated cells. Immunoblotting of cell lysates with anti-*Actn* antibody showed significant depletion of α-actinin (Figure 4), confirming that *Actn* dsRNA has been successfully delivered into the cells.

To test whether cell treatment with dsRNA affects the phosphorylation status of R2D2, VIG or Ago2a, we isolated the proteins from stable cell lines incubated with or without *Actn* dsRNA. The intake of dsRNA by S2 cells is a relatively fast process, resulting in degradation of cog-

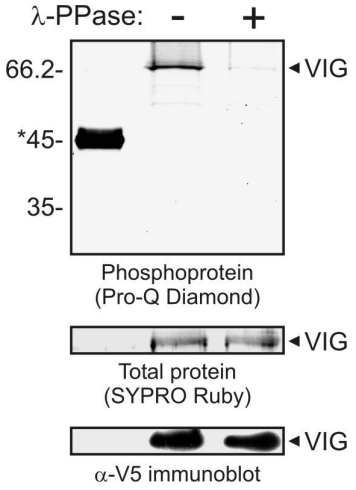

Figure 3. VIG dephosphorylation with λ-PPase. The protein was isolated from the VIG-expressing cell line, treated (+) or untreated (-) with λ-PPase, resolved by SDS-PAGE and subjected to specific phosphoprotein staining. Ovalbumin was used as a positive staining control (marked with an asterisk). The size of molecular mass standards (in kilodaltons) is indicated at left. The loading controls are shown in lower panels.

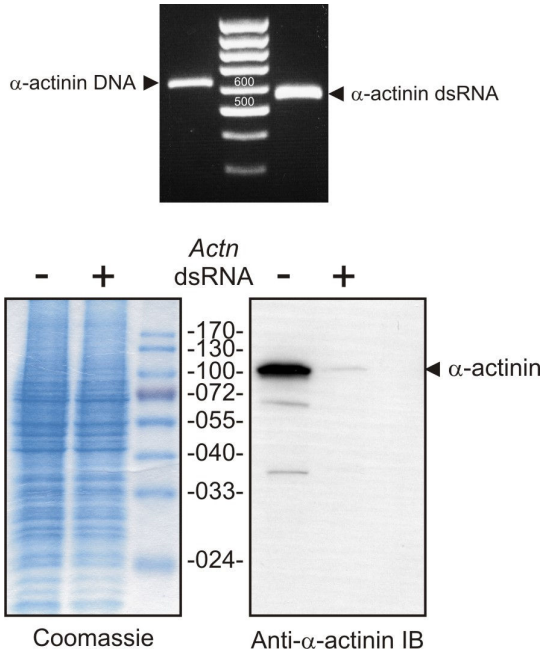

Figure 4. Preparation of *Actn* dsRNA and specific protein depletion. The upper panel shows a PCR fragment of ~600 nt corresponding to the largest exon of the α-actinin gene flanked by T7 RNA polymerase minimum binding sites. The PCR fragment was used as a template for *in vitro* transcription to synthesize the corresponding dsRNA. Five days after cell treatment with *Actn* dsRNA, total cell lysates were subjected to immunoblotting with anti-α-actinin antibody. Gel staining with Coomassie was used as a loading control. The sizes of marker proteins are indicated in kilodaltons.

nate mRNA within 24 hr (Dubrovsky et al, 2004). Subsequent protein depletion usually takes 1-5 days, depending on the protein turnover rate. Therefore, cell incubations with *Actn* dsRNA were carried out for 18 hr, enough to introduce dsRNA into the cells, but not enough to significantly deplete α-actinin. This helped minimize possible adverse effects of α-actinin depletion on target protein phosphorylation. Staining with Pro-Q Diamond and SYPRO Ruby revealed that the phosphorylation state of VIG, R2D2 and Ago2a remained unchanged following cell incubation with dsRNA. Similar phosphorylation levels were observed for VIG (Figure 2A, lane 5), while neither R2D2 nor Ago2a became phosphorylated (Figures 2A and 2B, lanes 3). The absence of Ago2a phosphorylation in dsRNA-treated cells was also confirmed by radioactive orthophosphate labeling (Figure 2C, lane 3). Thus, treatment of S2 cells with dsRNA had no immediate effect on VIG phosphorylation and was unable to activate phosphorylation of R2D2 or Ago2a.

VIG is efficiently phosphorylated by purified protein kinase C on multiple sites

The present finding that VIG is a phosphoprotein prompted us to perform a homology search in an attempt to find similar phosphoprotein(s) in other organisms. The search revealed that VIG shares significant sequence similarity with a human phosphoprotein Ki-1/57 (Figure 5).

Ki-1/57 (alternatively named HABP4) belongs to an evolutionarily conserved protein family present in organisms ranging from yeast to mammals. It has been long known that Ki-1/57 coimmunoprecipitates with a serine/threonine protein kinase activity (Hansen et al, 1990). On this basis, it was originally suggested that Ki-1/57 is a protein kinase, but cloning of its cDNA revealed the absence of any kinase domains in the protein sequence (Kobarg et al, 1997; Huang et al, 2000). Recently, the kinase activity associated with Ki-1/57 has been attributed to protein kinase C (PKC), and the PKC-mediated phosphorylation of Ki-1/57 has been demonstrated *in vitro* and *in vivo* (Nery et al, 2004). Therefore, we next sought to determine whether VIG could also be phosphorylated by PKC. To this end, lysates of VIG-expressing cells were immunoprecipitated with anti-V5 antibodies and subjected to *in vitro* kinase assays with purified rat brain PKC. Figure 6A, lane 2 shows that immunoprecipitated VIG was readily phosphorylated by PKC. As expected, VIG phosphorylation was reversed by phosphatase treatment (Figure 6A, lane 1), and no phosphorylated band corresponding to VIG was detected in immunocomplexes obtained from wild type cells (Figure 6A, lane WT). As a next step, we analyzed PKC-mediated phosphorylation of VIG by two-dimensional tryptic phosphopeptide mapping. We observed more than ten radioactive spots on a tryptic peptide map (Figure 6B), indicating that VIG is phosphorylated by PKC *in vitro* on multiple sites.

DISCUSSION

The major finding of the present study is that VIG, a component of the RNA-induced silencing complex, is phosphorylated *in vivo*. To our knowledge, this is the first report showing that the Vasa intronic gene product is a phosphoprotein. Of the three proteins tested, only VIG was

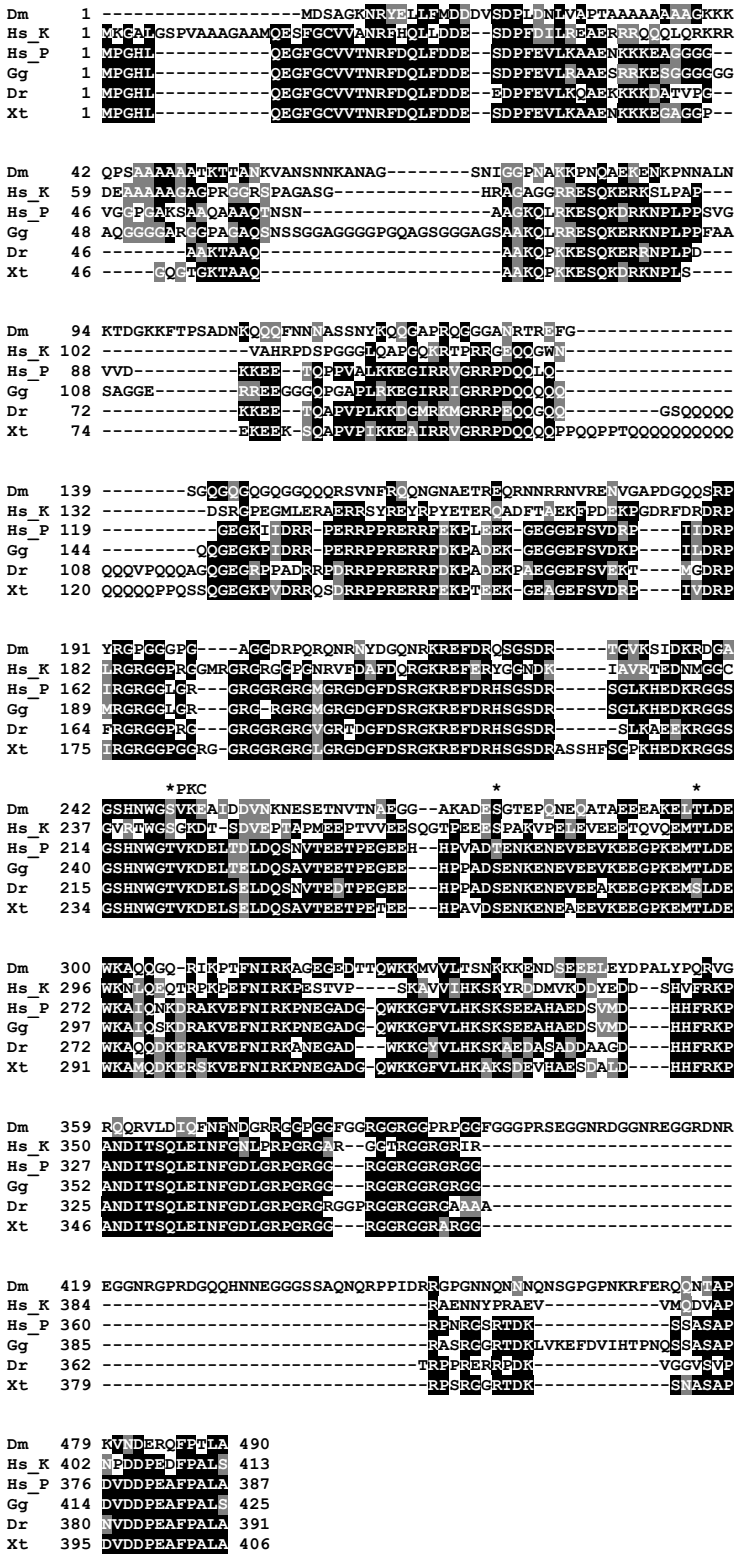

Figure 5. Multiple sequence alignment of the Drosophila VIG with its vertebrate homologs. Abbreviations: Dm, *Drosophila melanogaster*; Hs_K, *Homo sapiens* Ki-1/57; Hs_P, *Homo sapiens* PAI-RBP1; Gg, *Gallus gallus*; Dr, *Danio rerio*; Xt, *Xenopus tropicalis*. The alignment was produced with the ClustalW server at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw). Amino acid identity and similarity are indicated in black and grey shading, respectively. Asterisks indicate fully conserved putative phosphorylation sites predicted by the NetPhos 2 0 server (http://www.cbs.dtu.dk/services/NetPhos).

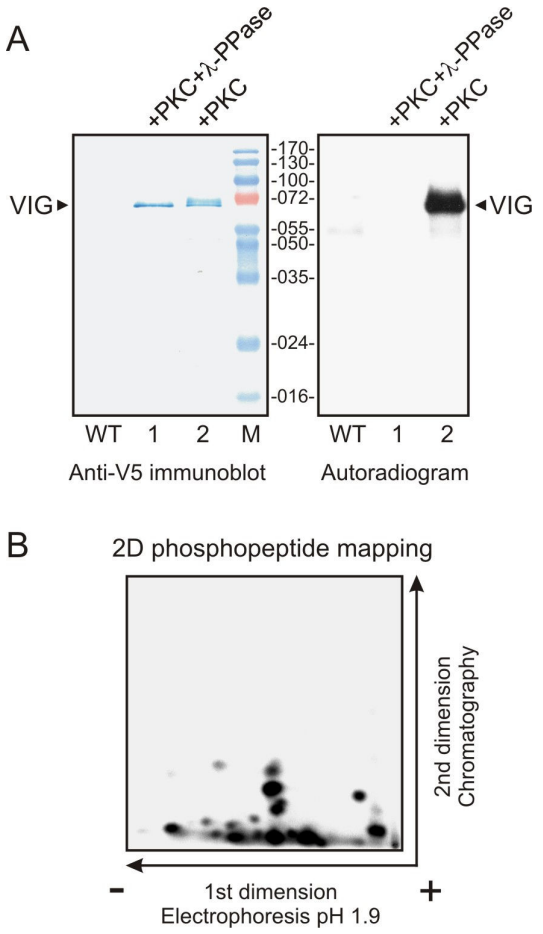

Figure 6. *In vitro* phosphorylation of VIG by PKC. **(A)** Immunoprecipitated VIG was assayed for phosphorylation by purified rat brain PKC in the presence of [γ-^{32}P]ATP. The reaction mixtures were treated (lane1) or not (lane 2) with λ-PPase. Material precipitated from wild type (WT) S2 cells was used as a negative control. Proteins were separated by 12% (w/v) SDS-PAGE and immunoblotted with anti-V5 antibody, and phosphorylation was detected by autoradigraphy. **(B)** The radiolabeled, phosphorylated VIG was transferred to a membrane, digested with trypsin and released peptides were separated by thin layer electrophoresis in the first dimension and chromatography in the second. Shown is the autoradiogram of the dried cellulose plate.

phosphorylated in S2 cells. The presence of identical V5-His tags in all three proteins ruled out that VIG could be artificially phosphorylated within the tag sequence. Because we were unable to obtain sufficient amounts of the full-length Ago2, we examined the phosphorylation status of its truncated form Ago2a. The protein retained the conserved and functionally important PAZ and PIWI domains, but lacked the N-terminal glutamine-rich domain. This domain, composed largely of glutamine residues, also has a number of serines, threonines, tyrosines and histidines that may potentially become phosphorylated. Phosphorylation of these residues, however, is unlikely to be important for Ago2's role in RNAi, since the glutamine-rich domain is not well conserved among the Argonaute family members. Nonetheless, a degree of caution should be exercised when extrapolating the results obtained for Ago2a to the full-length protein.

In this work, we demonstrate that VIG can be efficiently phosphorylated by mammalian PKC *in vitro*. A similar kinase is responsible for the *in vivo* phosphorylation of the VIG homolog Ki-1/57 (Nery et al, 2004). Therefore, members of the *Drosophila* PKC family (Shieh et al, 2002) may be suggested as candidate kinases for VIG phosphorylation in S2 cells. We observed no change in the phosphorylation state of VIG after cell treatment with dsRNA. This result is consistent with the possible involvement of PKC, a dsRNA-independent kinase, in the phosphorylation of VIG. A search with NetPhos 2.0 (Blom et al, 1999) revealed 17 potential phosphorylation sites in VIG, three of which are fully conserved between VIG, Ki-1/57 and other similar vertebrate proteins (Figure 5). Analysis of the VIG sequence with ScanProsite (Swiss Institute of Bioinformatics; http://www.expasy.ch/tools/scanprosite) showed that it contains at least three putative PKC phosphorylation motifs, and one of them is fully conserved in vertebrates. The presence of several PKC phosphorylation sites in VIG was confirmed by tryptic phosphopeptide mapping of VIG phosphorylated by PKC *in vitro*. However, it remains to be determined whether these sites are indeed phosphorylated *in vivo*.

Several lines of evidence indicate that VIG is a protein component of the RNAi pathway. VIG associates with RISC, binds the RISC nuclease Ago2 and co-precipitates with siRNAs (Caudy et al, 2002; Pham et al, 2004). The protein is required for efficient RNAi in S2 cells, as demonstrated by the 50% decrease in silencing efficiency caused by VIG suppression (Caudy et al, 2002). However, the exact function of VIG remains unclear. In this study, we found that VIG is similar to the human phosphoprotein Ki-1/57. Based on the functions of interacting partners and the protein localization, Ki-1/57 and another member of the VIG protein family, PAI-RBP1 (Heaton et al, 2001), have been proposed to be involved in chromatin remodeling and transcriptional regulation (Nery et al, 2004). Interestingly, both of these processes are implicated in transcriptional gene silencing (TGS). TGS is a form of dsRNA-induced silencing that leads to targeted chromatin modification and transcriptional shutdown (Almeida and Allshire, 2005). It serves as a natural defense against transposons and participates in cellular programs of gene expression and development. Both RNAi and TGS pathways recruit RISC effector complexes and share common components such as Dicer and Argonaute. Despite rapid progress in understanding the molecular mechanism of TGS, many questions remain about the identities and functions of the pathway components responsible for chromatin silencing in metazoans. The similarity between the known RISC component VIG and Ki-1/57, a protein with a suggested role in chromatin remodeling and transcriptional regulation, raises an intriguing possibility that VIG may be involved in TGS.

Reversible phosphorylation is arguably one of the most important means of regulating protein function. Therefore, it is tempting to speculate that VIG function in RISC might be regulated by phosphorylation. To address this

possibility, it would be necessary to have a better understanding of the molecular details of VIG phosphorylation. Further work is required to map the phosphorylation sites in VIG and determine which of them are functionally significant. The identity of the protein kinase(s) involved in VIG phosphorylation also remains to be established. Analysis of VIG phosphorylation in cells treated with PKC activators, inhibitors or PKC dsRNA may help determine whether VIG is a true substrate for PKC *in vivo*. Thus, the present study provides a starting point for future research on VIG phosphorylation and its possible role in RNAi and related processes.

CONCLUSIONS

- VIG, a component of the RNA-induced silencing complex, is a target for phosphorylation *in vivo*.
- Cell treatment with exogenous dsRNA has no effect on the phosphorylation state of VIG, indicating that dsRNA-independent kinase(s) are responsible for its phosphorylation.
- VIG shares sequence similarity with the human phosphoprotein Ki-1/57, a known substrate for protein kinase C (PKC).
- VIG is efficiently phosphorylated by PKC *in vitro*, suggesting PKC as a candidate kinase for VIG phosphorylation *in vivo*.

ACKNOWLEDGEMENTS

This work was supported by grants from the Academy of Finland (Grant Nos. 53862 and 206870). We thank O. Samuilova for kindly providing λ-PPase, A. Golubtsov for phosphatidylserine and G. Wahlström for anti-α-actinin antibody. We also thank K.-R. Hurme for assistance with radioactive labeling. We are grateful to K. Mäkeläinen for critical reading of the manuscript.

STATEMENT OF COMPETING INTERESTS

The authors declared no competing interests.

LIST OF ABBREVIATION

RISC: RNA-induced silencing complex
S2: Schneider 2
Ago2: Argonaute2
PKC: Protein kinase C
VIG: Vasa intronic gene product
dsRNA: Double-stranded RNA

REFERENCES

Almeida R and Allshire RC. 2005. RNA silencing and genome regulation. Trends Cell Biol, 15, 251-258.

Bernstein E, Caudy AA, Hammond SM and Hannon GJ. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature, 409, 363-366.

Blom N, Gammeltoft S and Brunak S. 1999. Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. J Mol Biol, 294, 1351-1362.

Carmell MA, Xuan Z, Zhang MQ and Hannon GJ. 2002. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev, 16, 2733-2742.

Caudy AA, Myers M, Hannon GJ and Hammond SM. 2002. Fragile X-related protein and VIG associate with the RNA interference machinery. Genes Dev, 16, 2491-2496.

Caudy AA, Ketting RF, Hammond SM et al. 2003. A micrococcal nuclease homologue in RNA effector complexes. Nature, 425, 411-414.

Ceman S, O'Donnell WT, Reed M, Patton S, Pohl J and Warren ST. 2003. Phosphorylation influences the translation state of FMRP-associated polyribosomes. Hum Mol Genet, 12, 3295-3305.

Cerutti L, Mian N and Bateman A. 2000. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends Biochem Sci, 25, 481-482.

Clemens JC, Worby CA, Simonson-Leff N et al. 2000. Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. Proc Natl Acad Sci USA, 97, 6499-6503.

Dubrovsky EB, Dubrovskaya VA, Levinger L, Schiffer S and Marchfelder A. 2004. Drosophila RNase Z processes mitochondrial and nuclear pre-tRNA 3' ends in vivo. Nucleic Acids Res, 32, 255-262.

Hammond SM, Boettcher S, Caudy AA, Kobayashi R and Hannon GJ. 2001. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science, 293, 1146-1150.

Hansen H, Bredfeldt G, Havsteen B and Lemke H. 1990. Protein kinase activity of the intracellular but not of the membrane-associated form of the Ki-1 antigen (CD30). Res Immunol, 141, 13-31.

Heaton JH, Dlakic WM, Dlakic M and Gelehrter TD. 2001. Identification and cDNA cloning of a novel RNA-binding protein that interacts with the cyclic nucleotide-responsive sequence in the Type-1 plasminogen activator inhibitor mRNA. J Biol Chem, 276, 3341-3347.

Huang L, Grammatikakis N, Yoneda M, Banerjee SD and Toole BP. 2000. Molecular characterization of a novel intracellular hyaluronan-binding protein. J Biol Chem, 275, 29829-29839.

Ishizuka A, Siomi MC and Siomi H. 2002. A Drosophila fragile X protein interacts with components of RNAi and ribosomal proteins. Genes Dev, 16, 2497-2508.

Ivanov KI, Puustinen P, Gabrenaite R et al. 2003. Phosphorylation of the potyvirus capsid protein by protein kinase CK2 and its relevance for virus infection. Plant Cell, 15, 2124-2139.

Kobarg J, Schnittger S, Fonatsch C et al. 1997. Characterization, mapping and partial cDNA sequence of the 57-kD intracellular Ki-1 antigen. Exp Clin Immunogenet, 14, 273-280.

Lee YS, Nakahara K, Pham JW et al. 2004. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell, 117, 69-81.

Liu Q, Rand TA, Kalidas et al. 2003. R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. Science, 301, 1921-1925.

Liu J, Carmell MA, Rivas F et al. 2004. Argonaute2 is the catalytic engine of mammalian RNAi. Science, 305, 1437-1441.

Meister G and Tuschl T. 2004. Mechanisms of gene silencing by double-stranded RNA. Nature, 431, 343-349.

Nery FC, Passos DO, Garcia VS and Kobarg J. 2004. Ki-1/57 interacts with RACK1 and is a substrate for the phosphorylation by phorbol 12-myristate 13-acetate-activated protein kinase C. J Biol Chem, 279, 11444-11455.

Otey CA and Carpen O. 2004. α-actinin revisited: a fresh look at an old player. Cell Motil Cytoskeleton, 58, 104-111.

Pham JW, Pellino JL, Lee YS, Carthew RW and Sontheimer EJ. 2004. A Dicer-2-dependent 80S complex cleaves targeted mRNAs during RNAi in Drosophila. Cell, 117, 83-94.

Shieh BH, Parker L and Popescu D. 2002. Protein kinase C (PKC) isoforms in Drosophila. J Biochem (Tokyo), 132, 523-527.

Siomi MC, Higashijima K, Ishizuka A and Siomi H. 2002. Casein kinase II phosphorylates the fragile X mental retardation protein and modulates its biological properties. Mol Cell Biol, 22, 8438-8447.

Song JJ, Smith SK, Hannon GJ and Joshua-Tor L. 2004. Crystal structure of Argonaute and its implications for RISC slicer activity. Science, 305, 1434-1437.

Sontheimer EJ. 2005. Assembly and function of RNA silencing complexes. Nat Rev Mol Cell Biol, 6, 127-138.

Steinberg TH, Agnew BJ, Gee KR et al. 2003. Global quantitative phosphoprotein analysis using multiplexed proteomics technology. Proteomics, 3, 1128-1144.

Tomari Y, Matranga C, Haley B, Martinez N and Zamore PD. 2004. A protein sensor for siRNA asymmetry. Science, 306, 1377-1380.

Zamore PD, Tuschl T, Sharp PA and Bartel DP. 2000. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell, 101, 25-33.

SHORT COPYRIGHT STATEMENT