Phosphorylation of human Argonaute proteins affects small RNA binding

Sabine Rüdel[1], Yanli Wang[2], René Lenobel[3,4], Roman Körner[3], He-Hsuan Hsiao[5], Henning Urlaub[5], Dinshaw Patel[2], Gunter Meister[1]*

Supplementary information

Supplementary Methods

Plasmids and mutagenesis

Plasmids encoding F/H-tagged as well as myc_6-tagged proteins were described previously (Rudel et al., 2008). Point mutations were introduced into human Ago2 coding sequence by two PCR reactions using the following desoxy-oligonucleotides. Mutated codons are underlined: Y529A_fw: 5'-gcccggcaagacgcccgtggcagccgagg; Y529F_fw: 5'-gcccggcaagacgcccgtgttcgccgagg; Y529Q_fw: 5'-gcccggcaagacgcccgtgcaggccgagg; Y529E_fw: 5'-gcccggcaagacgcccgtggaggccgagg; Ago2_rev_AscI: 5'-atctggcgcgccttatcaagcaaagtacatggtgcgcagagtg. The first PCR generated mutated 3' fragments of Ago2 that were subsequently used as reverse primers together with Ago2_fwd_FseI: 5'aaaggccggccatgtactcgggagccggcc. The resulting mutated full-length Ago2 coding sequence was digested with FseI and AscI and cloned into a modified pIRESneo vector (Meister et al., 2004).

Cell culture and transfection

In this study only HeLa and HEK 293 cells have been used and only the latter ones were transfected by calcium phosphate procedure. Cell maintenance and transfection by calcium phosphate procedure were described previously (Rudel et al., 2008).

Total extract preparation, immunoprecipitation and Western blotting

Total cell extracts were prepared as described before (Rudel et al., 2008), but in the presence of sodium orthovanadate (Sigma-Aldrich) that has been treated with H_2O_2 before. Immediately before lysis, cells have been incubated with phosphatase inhibtor for 10 minutes at 37°C in a final concentration of 1 mM. Vanadate was also included in the lysis buffer.

Immunoprecipitation and Western blotting were performed as reported previously (Rudel et al., 2008). For immunoprecipitation either anti-FLAG M2 agarose beads or anti-myc agarose beads (both Sigma) were used. Monoclonal anti-HA antibody was purchased from HISS Diagnostics, Germany, monoclonal anti-myc antibody from Abcam, UK, peroxidase-conjugated secondary anti-mouse IgG antibody from Sigma and anti-rabbit IgG antibody from Jackson, West Grove, PA.

Immunofluorescence

Sample preparation has been described elsewhere (Rudel et al., 2008). Immunofluorescence was recorded using a TCS SP2 Confocal Laser Scanning microscope and Confocal Software softWoRx (Leica Microsystems, Germany). Monoclonal anti-LSm4 antibody was purchased from Geneway, FITC-conjugated

anti-chicken IgY antibody from Sigma and TexasRed-conjugated anti-mouse IgG antibody from Vector Laboratories.

For quantitative analysis of Ago2-Y529 mutants' localization to P-bodies (PBs), twelve z-section images of each sample were recorded and processed to maximum projections using confocal software. PBs were identified by the presence of LSm4. For each mutant 100 PBs were analyzed for co-localization of the Ago2 mutant of interest. The ratio of PBs positive for both proteins to the total number of PBs was determined for each cell. Standard deviation was calculated using the ratios obtained for the single cells.

Rescue experiments

HeLa cells were reverse transfected in 6 well plates with siRNAs at 65 nM final concentration using RNAiMAX (Invitrogen) according to the manufacturer's instructions. The following siRNAs were used (sense, antisense): Ago2-3'UTR siRNA, 5' r(CCGUCCCAGAUUUCAAACUU)dT, 5' r(AAGUUUGAAAUCUGGGACGG)dT; non-silencing control siRNA, 5' r(UUGUCUUGCAUUCGACUAAU)dT, 5' r(UUAGUCGAAUGCAAGACAAU)dT. SiRNAs have been synthesized as described before (Chen et al., 2008). After 3d, cells were seeded to 48 well plates and 6 hours later transfected with 400 ng of the appropriate VP5-Ag02-Y529 plasmid and 100 ng pMIR-RNL-TK-miR21 plasmid (Hock et al., 2007) using lipofectamin 2000 (Invitrogen). 2d later, cells were lysed using passive lysis buffer (Promega, USA). Luciferase activities were measured on a Mithras LB 940 luminometer (Berthold technologies, Germany). All samples were assayed in 3 replicates and each experiment was at least repeated three times. Firefly/renilla luminescence ratios were normalized to the

ratio recorded for the control, transfected with a non-silencing siRNA but no VP5 construct. pMIR-RNL-TK-miR21 encodes firefly luciferase under the control of a HSV-Tk promotor and harbors a site perfect complementary to miR-21 in the firefly luciferase 3'UTR. In addition this plasmid encodes renilla luciferase under the control of a SV40 promotor.

Tethering experiments

pCI-neo-HA, pCI-neo-N-HA and pRL-5BoxB plasmids were kindly provided by the Filipowicz laboratory (Pillai et al., 2004). Protein-coding sequences were inserted into pCI-neo-HA and pCI-neo-N-HA respectively using the EcoRI and NotI restriction sites.

HeLa cells were seeded to 48 well plates and 6 hours later transfected with 300 ng N-HA or HA fusion protein coding plasmids, 120 ng pRL-5BoxB and 80 ng pMIR-REPORTER (Ambion) using lipofectamin 2000 (Invitrogen). After 2d, cells were lysed and luciferase activity measured as described for rescue experiments. Renilla/firefly luminescence ratios of the tethered N-HA fusion proteins were normalized to the ratio recorded for the not tethered HA fusion proteins.

Mass spectrometry

Coomassie-stained protein bands were in-gel digested by trypsin (sequencing grade, Roche, Germany) essentially as described (Shevchenko et al., 1996) and phosphorylated peptides were enriched using TiO_2 affinity purification (Thingholm et al., 2006). Subsequently, tryptic peptide mixtures were separated on a CAPLC (Waters, USA) or an Ultimate (Dionex, Germany) nanoHPLC system directly coupled to a QTOF (Waters, UK) or an orbitrap (Thermo, Germany)

electrospray mass spectrometer. The mass spectrometers was operated in data dependent MS/MS mode to automatically switch between MS survey and MS/MS fragmentation scans of the four (QTOF) or five (orbitrap) most abundant precursor ions. Database searches were performed using the Mascot (Matrixscience, UK) software package and identified phosphorylation sites were further validated by visual inspection of MS/MS spectra.

References

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Supplementary Figure legends

Suppl. Figure 1: Multiple alignment of phosphorylated peptide sequences from different species.

		(1)	(2)
hsAgo2	NP_036286	242.....255	290.....307
hsAgo2	NP_036286	SIEEQQKPLTDSQR	SHQTFPLQQESGQTVECT
hsAgo1	NP_036331	NIDEQPKPLTDSQR	SHQTFPLQLESGQTVECT
hsAgo3	NP_079128	NIDEQPRPLTDSHR	SHQTFPLQLENGQTVERT
hsAgo4	NP_060099	NINEQTKPLTDSQR	SHQTFPLQLENGQAMECT
Hili	NP_001129193	AIYQQNK---EHFQ	PKDSFTMSDGK----EIT
Hiwi	NP_004755	HQTEEHK-----FQ	PKSTFKKADGS----EVS
Hiwi2	NP_689644	----QRTGLS----	PTHTF---QKRDG-TEIT
mmAgo2	NP_694818	SIEEQQKPLTDSQR	SHQTFPLQQESGQTVECT
rnAgo2	NP_067608	SIEEQQKPLTDSQR	SHQTFPLQQESGQTVECT
dmAgo1	NP_725342	DINEQRKPLTDSQR	QMQSFPLQLENGQTVECT
dmAgo2	NP_648775	AKINNTTNLDYSRR	SSETF---EHDGKKV--T
ceAlg2	NP_871992	ALAE-RRALSDAQR	QTQTFPLQLETGQTIECT
spAgo	NP_587782	NVRD----LTRFDL	S-DSFFVRRLNGEEQKIS
atAgo1	NP_849784	S-----RPLSDADR	RELTFPVDERNTQK---S

		(3)	(4)	(5)
hsAgo2	NP_036286	385.....395	526....533	796.....807
hsAgo2	NP_036286	SASFNT--DPYVR	TP-VYAE-VK	SVSIPAPAYYAH
hsAgo1	NP_036331	NASYNL--DPYIQ	TP-VYAE-VK	SVSIPAPAYYAR
hsAgo3	NP_079128	SANYET--DPFVQ	TP-VYAE-VK	SVSIPAPAYYAH
hsAgo4	NP_060099	SNSMVGGPDPYLK	TP-VYAE-VK	SVSIPAPAYYAR
Hili	NP_001129193	----------	DD-LYGA-IK	TIRVPAPCKYAH
Hiwi	NP_004755	HKNDNV--QRELR	KD-KYDA-IK	VIRVPAPCQYAH
Hiwi2	NP_689644	NARFEL--ETWGL	QK-TYYDSIK	IVSVPAPCQYAH
mmAgo2	NP_694818	SASFNT--DPYVR	TP-VYAE-VK	SVSIPAPAYYAH
rnAgo2	NP_067608	SASFNT--DPYVR	TP-VYAE-VK	SVSIPAPAYYAH
dmAgo1	NP_725342	RADFNN--DSYVQ	TP-VYAE-VK	SVSIPAPAYYAH
dmAgo2	NP_648775	YFQHNL--DPTIS	RI-SYDT-IK	SVSYPAPAYLAH
ceAlg2	NP_871992	KAELSA--DPFAH	TP-IYAE-VK	SVSIPAPAYYAH
spAgo	NP_587782	QMDWDT--DPYLT	SPEPYGS-IK	AVSLVPPVYYAH
atAgo1	NP_849784	LNDYAK--DNYAQ	NGSLYGD-LK	SVSIVPPAYYAH