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Supplemental Data

Human Cytomegalovirus Protein UL38 Inhibits Host Cell Stress Responses by Antagonizing the Tuberous Sclerosis Protein Complex

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Supplemental Experimental Procedures

Construction of Viruses with tagged proteins

BAD*in*UL38TAP was prepared by modification of a BAC clone of BAD*wt*, pAD/Cre (Yu et al., 2002), in *E. coli* by using linear recombination (Borst et al., 2001). PCR amplification was used to generate a targeting cassette for recombination. The template for amplification contained a TAP tag followed by a kanamycin cassette flanked by FRT sites. PCR primers were as follow: 5'TCGTGTACACGGCCGGGGAGGGCGACGTGGTACAGATGGTGGTCGTGGTCGCCGGAAGAAGATGGAAAAAG 3' and 5'ACTACAAAAAAAAAAGCTGAACATGGTCATCTAGCAGCAAAGTTCTCCTTCGTCGTGGAATGCCTTCG3'. The underlined segments represent sequence homologous to the viral sequence at the 3' end of the UL38 ORF. After construction, the BAC DNA was purified, checked for genomic integrity by restriction digestion, and the TAP-tagged UL38 locus sequenced. UL38TAP BAC DNA was introduced into fibroblasts by electroporation to generate stocks of BAD*in*UL38TAP virus, and yields were determined by plaque assay on fibroblasts. Expression of UL38TAP protein after infection with the recombinant virus was confirmed by Western blot using anti-Protein A antibody (Sigma; data not shown). BAD*in*UL99GFP is a derivative of BAD*wt* containing a GFP tag fused to the C-terminus of the UL99 ORF, which was prepared using the same approach as for BAD*in*UL38TAP. BAD*in*UL99GFP replicates in fibroblasts with wild-type kinetics (data not shown).

Purification and identification of pUL38-interacting proteins by mass spectroscopy

Frozen HCMV-infected cell pellets were loaded in round-bottom eppendorf tubes with one 2 mm stainless steel grinding ball placed in the middle of the sample. The cryogenic lysis (Cristea et al., 2006) was performed in 6 steps of 3 min each at 30 Hz using the Retsch MM 301 Mixer Mill. The ground cell powder was homogenized in lysis buffer. Several buffers were tested to obtain an efficient protein extraction and maintain viral-host protein interactions. The optimized lysis buffer chosen for this study was 20 mM K-HEPES, pH 7.4, 110 mM KOAc, 2 mM $MgCl_2$, 0.1% Tween-20, 1% Triton, 0.25 M NaCl, 1/100 (v/v) protease inhibitor mixture (20 mg/mL PMSF + 0.4 mg/mL pepstatin A), and 1/200 (v/v) protease inhibitor cocktail (Sigma). M-270 Epoxy Dynabeads (Invitrogen) were coupled to IgG (MP Biomedicals) using 7 µg IgG/mg of beads (Cristea et al., 2005). Immunoaffinity purifications were performed by using the protein A tag, employing 1 h incubations of the cellular lysate with conjugated magnetic beads (Cristea et al., 2006; Cristea et al., 2005). Isolated proteins were resolved by electrophoresis on a 4-12% NuPAGE Novex Bis-Tris pre-cast 1-DE gel

(Invitrogen), and stained with Coomassie blue (Pierce). The entire gel lane was cut into 1-2 mm pieces and proteins were digested with trypsin (Promega). The resulting peptides were extracted on reverse phase resin (Poros 20 R2; PerSeptive Biosystems) and subjected to matrix-assisted laser desorption (MALDI) mass spectrometric analysis (Cristea et al., 2004). An in-house built MALDI interface was coupled to either a QqToF instrument (QqTOF Centaur, MDS Sciex) for peptide fingerprinting (MS) (Krutchinsky et al., 2000) or an ion trap (LCQDECAXP^{PLUS}, Finnigan, Thermo Fisher Scientific) for amino acid sequence analysis (MS/MS) (Krutchinsky et al., 2001). XProteo (http://www.xproteo.com) and ProFound (http://prowl.rockefeller.edu) algorithms (Zhang and Chait, 2000) were used to correlate peptide mass fingerprint data and tandem MS CID data with human and viral databases to identify proteins (Cristea et al., 2004). Only proteins confirmed by MS/MS analyses were included in the results.

Protein analysis

For immunoprecipitation experiments (Wang and Shenk, 2005), frozen cell pellets were resuspended in 1 ml RIPA buffer (Tris-HCl, 50 mM, pH 7.4; 1% NP-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA). Lysates were sonicated and incubated on ice for 30 min with vortexing for 5 sec every 5 min. Insoluble material was pelleted by centrifugation at 16,000 x g for 5 min at 4°C. Lysates were precleared for 30 min with Protein A/G Sepharose with rotation at 4°C. Antibodies were added, and the sample was incubated for 1 h at 4°C with rotation. Protein A/G Sepharose (Santa Cruz) in RIPA buffer was added, and the sample was incubated for 1 h at 4°C with rotations. The Sepharose was pelleted and washed 5 times with RIPA buffer. Following the final wash, residual buffer was removed and the sepharose was resuspended in sample buffer, boiled at 100°C for 5 min, and insoluble material pelleted by spinning for 3 min at room temperature at 16,000 x g. Protein concentrations were determined using the Bradford assay (Bio-Rad). Samples were resolved on 10% SDS-containing polyacrylamide gels, and proteins were identified by Western blot.

Western blots assays (Munger et al., 2006) were performed with rabbit polyclonal antibodies in 1X TBST buffer (1X TBS, 0.1% Tween 20) with 5% bovine serum albumin (BSA) overnight at 4°C with rocking. Western blots were preformed with mouse monoclonal antibodies in 1X TBST with 1% BSA for 1 h at room temperature. Appropriate secondary antibody was used at 1:5000 dilution in 1X TBST plus 1%BSA for 1 h at room temperature. Antibody complexes on blots were visualized using ECL Reagent (GE Healthcare Bio-Sciences).

For immunofluorescent analysis (Sharon-Friling et al., 2006), fibroblasts were grown to confluence on glass coverslips in six-well plates. At 24 h after infection with HCMV at 0.01 PFU/cell, cells were washed in PBS, fixed for 15 min in 2% paraformaldehyde, and permeabilized for 15 min in 0.1% Triton X-100. After washing with PBS-T (PBS containing 0.2% Tween 20), the cells were incubated for 30 min in PBS-blocking buffer (PBS containing 2% bovine serum albumin and 0.2% Tween 20) and incubated with either a mouse or rabbit primary antibody in PBS blocking buffer for 1 h at room temperature. After further washing with PBS-T, slides were incubated for 30 min at room temperature with either goat anti-mouse or goat-anti rabbit immunoglobulin Alexa 488, 546 or 633 (Invitrogen). The second antibody was supplemented with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) and Lectin HPA (Invitrogen). Cells were washed with PBS-T, mounted and viewed using a Zeiss LSM510. Quantitative estimates of protein colocalization (Costes et al., 2004) were performed using the Velocity software package (Improvision).

Supplemental References

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Table S1. Proteins captured by pUL38TAP[a]		gi #	# peptides from MS	% Sequence coverage	XProteo score	MS/MS confirm	# peptides confirmed	Peptides confirmed by MS/MS
Tag	CTAP-tag [Cloning vector pUAST-CTAP]	54306511	12	77	3.1	Y	2	TAALAQHDEAVDNKFNK NFIAVSAANR
Viral HCMV	UL38	1780815	7	19	7.4	Y	2	IPMTFVDR AILESER
	UL29	29423462	16	32	13.3	Y	2	LQPNVPLVHPDHR YESIGPVDR
	UL28	28373247	6	16	2.9	Y	2	DYVDEIVEGAWFK HTFAGMYELSQILHDR
	UL52	52139239	8	12	5.8	N	n/a	n/a
Host Homo Sapiens	Tuberin	3522922	49	28	22.9	Y	4	LESQAGQQVSR HSYTLPIASSIR DGWIQNLQALMER ESSAAEAFR
	PREDICTED: ubiquitin specific protease 24	51458553	39	17	22.3	Y	2	EFDYLPPVDSR VYDQTNPYTDVR
	PREDICTED: similar to ubiquitin A-52 residue ribosomal protein fusion product 1	51467509	9	25	n/o	N	n/a	n/a
	similar to Ras GTPase-activating protein 4 (RasGAP-activating-like protein 2)	89026715	5	10	3.4	Y	2	YVELDPSK DELDLQR
	ubiquitin specific protease 9	2580558	18	5	8.5	Y	4	DGLFDTIQR ELLAFQTSEK IELFVGGELIDSEDDR ALTLQDLDNIWAAQAGK
	Chromodomain helicase-DNA-binding protein 4 (CHD-4) (Mi-2 autoantigen 218 kDa protein) (Mi2-beta)	5921744	46	23	19.9	Y	5	APEPTPQQVAQQQ LLEQALVIEEQLR* ENEFSFEDNAIR QELDDILK
	Chromodomain helicase-DNA-binding protein 3 (CHD-3) (Mi-2 autoantigen 240 kDa protein) (Mi2-alpha)	88911273	31	11	8.6	Y	6	MPDKDDIR RDSEEEFGSER LPSGAYEGGALIKSSGK NFEALNSR QSYWRHR LLEQALVIEEQLR*
	chromodomain helicase DNA binding protein 5	56417878	18	11	4.5	Y	3	IYDIWHR LLEQALVIEEQLR* VGGNIEVLGFNAR*

	HDAC1	49456395	7	41	8.7	Y	4	YGEYFPGTGDLR* YYAVNYPLR NSSNFKK KEVTEEEK
	HDAC2	55961192	12	24	10.8	Y	2	YGEYFPGTGDLR* YHSDEYIKFLR
	MYST histone acetyltransferase (monocytic leukemia) 3	5803098	18	7	6.3	Y	4	KPFLHRR QSLNGPYMR LNTKATNVDGK DKPVAEPIPICSFCLGTK
	MTA1	2498589	12	16	5	Y	2	QIDQFLVVAR* YLETHPRPPKPDPVK
	MTA2	4126710	23	39	4.2	Y	3	QIDQFLVVAR* LNPADAPNPVVFVATK TLLADQGEIR
	‡Chromatin assembly factor 1 subunit C (Retinoblastoma-binding protein 4) (RBBP-4) (RBAP48)	1172846	12	30	12.2	Y	4	VINEEYK* INHEGEVNR* RLNVWDLSK* TPSSDVLVFDYTK*
	‡Retinoblastoma-binding protein 7 (RBBP-7) (Nucleosome remodeling factor subunit RBAP46)	2494891	7	28	1.2	Y	4	VINEEYK* INHEGEVNR* RLNVWDLSK* TPSSDVLVFDYTK*
	Zinc finger protein 420	20072764	11	21	4.5	N	n/a	n/a
	MAP6 protein	39795300	14	21	9.3	Y	1	DQGSVVPESLK
	microtubule-associated protein 6 isoform 1	48375173	n/o	n/o	n/o	Y	2	DQGPTVLQPPKNQGR GQGPMVQEPLKK
	microtubule-associated protein 6 isoform 2	48375167	8	27	n/o	Y	1	GEASKPTTANDK
	TBL2 protein	15277891	10	20	8.4	N	n/a	n/a
	annexin A2 isoform 1	50845388	11	30	n/o	Y	1	LSLEGDHSTPPSAYGSV K
	HNRPM protein	33874022	6	12	n/o	N	n/a	n/a
	HNRPH1	48145673	4	10	3.3	Y	3	YIEIFK VHIEIGPDGR HTGPNSPDTANDGFVR
	heterogeneous nuclear ribonucleoprotein U isoform b	14141161	n/o	n/o	n/o	Y	3	FIEIAAR SGKNQFNR DLPEHAVLK
	71 Kd heat shock cognate protein	32467	11	18	n/o	Y	2	TTPSYVAFTDTER GTLDPVEKALR
	nebulette	3660517	11	35	n/o	N	n/a	n/a

	MAP3K7	56204061	6	16	n/o	Y	3	TNKHYSSATW QIESESER SFAGTVAWMAPEVIR
	WDR66 protein	23271967	n/o	n/o	n/o	Y	3	GDLFVLR RVSDIQSK VKLFNATTK
	golgi antigen gcp372	808869	39	12	7.7	N	n/a	n/a
	GATA zinc finger domain containing 2B = transcription repressor p66	21218438	8	16	7.8	Y	2	SLDPADERDDVLAK SLDPADER
	Golgin subfamily A member 4 (Trans-Golgi p230) (256 kDa golgin) (Golgin-245)	12643718	27	8	n/o	Y	5	ELENTALELSQK EIEILTQK LLDQEAK DAKNLIEQLEQDK EREVHILEEK
	Histone H2A.5	29553970	3	27	0.9	Y	3	AGLQFPVGR VTIAQGGVLPNIQAVLLP K HLQLAIR
	Histone H2B	1568557	5	33	3.2	Y	2	LLLPGELAK KESYSVYVYK
	Histone H4	48145673	5	40	n/o	Y	1	VFLENVIR
Likely contam-inants Homo Sapiens	52kD Ro/SSA autoantigen	14790039	13	31	7.3	Y	1	LQVALGELR
	Lamin A protein	386856	19	27	11.1	Y	2	EELDFQKNIYSEELR MQQQLDEYQELLDIK
	myosin, heavy polypeptide 9, non-muscle	12667788	39	22	18.6	Y	7	EAPSPEKTLPPQK IAEFTTNLTEEEEKSK EQADFAIEALAKATYER DVLLQVDDERR VVFQEFR DLGEELEALK VSHLLGINVTDFTR
	Vimentin	37852	36	65	15.2	Y	3	LQEEMLQR FADLSEAANR SLYASSPGGVYATR
	ACTB protein	15277503	14	50	5.1	Y	1	AGFAGDDAPR
	SSA1	14994115	7	16	n/o	Y	1	LQVALGELR
	Ig gamma heavy chain constant region	2136983	7	43	n/o	N	n/a	n/a

[a]The majority of listed proteins were first detected by MS analysis, and then confirmed through MS/MS analyses. Several proteins were not observed (n/o) at the MS analysis stage, or did not obtain a score from the MS analysis (not available; n/a), but were identified from the MS/MS analyses. The Xproteo scores obtained for the MS analyses are indicated. The score is calculated in two

steps. First, candidate proteins are ranked by probability scores calculated from an improved version of the Bayesian algorithm used in ProFound (71). Second, for each candidate protein the discriminability (d') score is calculated, illustrated in the table as the final XProteo score. Discriminability is defined as the normalized distance between the Gaussian score distributions of the proteins identified with high scores and the randomly matched proteins in units of standard deviation of the score distributions. The peptides confirmed by MS/MS analyses are indicated for each protein. Asterisks designate peptides common to several isoforms of the same protein, identified from different bands on the gel. However, the presence of the various shown isoforms was confirmed through MS/MS analyses of unique peptides. ‡, because of the similarity of the sequences of RbAp48 and RBAp46, either or both may be present.