Biochemical and Biophysical Research Communications 472 (2016) 539–544

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Proteomic insight into the effects of the *Salmonella* ubiquitin ligase SlrP on host cells

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ARTICLE INFO

Article history:
Received 23 February 2016
Accepted 6 March 2016
Available online 8 March 2016

Keywords:
SlrP
Salmonella
Type III secretion
iTRAQ
Cell adhesion

ABSTRACT

The virulence of the human and animal pathogen *Salmonella enterica* serovar Typhimurium is dependent on two type III secretion systems. These systems translocate proteins called effectors into eukaryotic host cells. SlrP is a *Salmonella* type III secretion effector with ubiquitin ligase activity. Here, we used two complementary proteomic approaches, two-dimensional gel electrophoresis and iTRAQ (isobaric tags for relative and absolute quantification) to study the consequences of the presence of SlrP in human epithelial cells. We identified 37 proteins that were differentially expressed in HeLa cells expressing *slrP* compared to control cells. Microarray analysis revealed that more than a half of differentially expressed proteins did not show changes in the transcriptome, suggesting post-transcriptional regulation. A gene ontology overrepresentation test carried out on the differentially expressed proteins revealed enrichment of ontology terms related to several types of junctions mediating adhesion in epithelial cells. Consistently, *slrP*-transfected cells showed defects in migration and adhesion. Our results suggest that the modification of cell–cell interaction ability of the host could be one of the final consequences of the action of SlrP during an infection.

1. Introduction

Salmonella are Gram negative bacteria belonging to the family Enterobacteriaceae. The species *Salmonella enterica* is divided into several subspecies and many serovars [1]. Some of the serovars belonging to the subspecies *enterica* are pathogenic for humans and other warm-blooded animals causing different diseases, from enteritis to typhoid fever, depending on the specific serovar–host combination. More than 500000 human deaths occur each year worldwide as a consequence of these diseases [2,3].

Salmonella and many other Gram negative pathogens rely on type III secretion systems (T3SS) for their relationships with the host cells [4]. These systems are complex structures that form a sort of molecular needle that spans the inner membrane, the periplasmic space and the outer membrane of the bacteria, as well as the cell membrane of the host. They deliver effector proteins directly into eukaryotic cells. Bacteria of the species *S. enterica* posses two virulence-associated T3SS that are able to secrete more than 30 effectors [5]. The structural components and some of the effectors of these two systems are encoded in two different *Salmonella* pathogenicity islands, SPI1 and SPI2, respectively [6–8]. The SPI1-related system, T3SS1, is necessary for the internalization of *Salmonella* into host cells using a mechanism characterized by the formation of large membrane ruffles at the bacterial entry site [9]. This mechanism involves actin remodeling and membrane fusion induced by specific *Salmonella* effectors. T3SS1 effectors have also a role in the disruption of epithelial tight junctions [10,11] and in the induction of different forms of cell death in epithelial cells and macrophages [12]. Once inside the host cell, *Salmonella* resides in a specific niche known as *Salmonella*-containing vacuole (SCV). The environmental conditions found in this compartment induce the

Abbreviations: T3SS, type three secretion system; SPI, *Salmonella* pathogenicity island; SCV, *Salmonella*-containing vacuole; iTRAQ, isobaric tags for relative and absolute quantification; HEMA, 2-hydroxyethyl methacrylate; GO, gene ontology.

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http://dx.doi.org/10.1016/j.bbrc.2016.03.014

expression of the SPI2-related secretion system, T3SS2. This system delivers more than 20 effectors through the vacuole membrane into the host cytosol [13]. These effectors, together with a subset of T3SS1 effectors, are involved in the biogenesis, maturation and maintenance of the SCV [14]. An important host process that is exploited by *Salmonella* through several T3SS effectors is the ubiquitination system: some T3SS effectors are ubiquitination substrates, act as E3 ubiquitin ligases or mimic deubiquitinases [15].

One of the effectors involved in the manipulation of the ubiquitination system is SlrP [16]. This is a protein of 765 amino acids with three domains [17]: the N-terminal domain necessary for secretion through T3SS1 and T3SS2 [18]; the central domain with several copies of a leucine-rich repeat signature, a protein motif frequently involved in protein–protein interactions [19]; and the C-terminal domain (NEL domain), which is conserved in other effectors and possesses E3 ubiquitin ligase activity [20,21]. SlrP interacts with mammalian thioredoxin-1 (Trx1) and catalyzes ubiquitination of this substrate *in vitro* [20]. We recently solved the 3D structure of the SlrP-Trx1 complex and determined the Trx1 ubiquitination site [22]. In addition, SlrP can interact with the chaperone ERdj3 in the endoplasmic reticulum [23]. HeLa cells stably transfected with *slrP* are more prone to cell death and our experiments suggest that SlrP promotes death in host cells by interfering with the functions of its targets Trx1 and ERdj3 [20,23].

Proteomic profiles reflect better the actual physiological condition of an organism than the transcriptomic ones. This fact is leading to the implementation of these technologies to many fields of biological research. Here, we use two proteomic approaches to get insight into the global effect of SlrP on host cells. Previously published works have suggested the complementary nature of these methods [24].

2. Materials and methods

2.1. Cell culture and lysis

HeLa cells (ECAC No. 93021013) were cultured and lysed in NP40 buffer as previously described [20].

2.2. Two-dimensional gel electrophoresis

Proteins extracts for SlrP- and empty vector-transfected cells were prepared in triplicate and resolved by two-dimensional gel electrophoresis [25]. Protein detection was achieved with Coomassie Brilliant Blue R-250 (0.1% w/v in methanol 50% v/v). Identification of differentially expressed proteins was achieved using mass spectrometric analysis at the Proteomics Facility of the University of Córdoba (Córdoba, Spain).

2.3. Isobaric tags for relative and absolute quantification (iTRAQ) analysis

HeLa cells (10^7 per cell line) were lysed as described in Supplementary materials and methods and 100 μg of proteins of every extract were labeled with the iTRAQ reagent according to the manufacturer's instructions (AB Sciex). The analysis was carried out at the Proteomics Facility of the University of Córdoba with the protocol described in Supplementary materials and methods.

2.4. RNA preparation and gene array processing

Total RNA from HeLa cells stably transfected with pcDNA3 or its derivative encoding the SlrP-3xFLAG fusion was isolated in triplicate and processed for hybridization to GeneChip Human Gene 1.0 ST Arrays (Affymetrix) at the Genomics Unit of the Andalusian Center for Molecular Biology and Regenerative Medicine (CABIMER, Seville, Spain) as previously described [26].

2.5. Immunoblot and antibodies

Proteins in cell extracts were resolved by SDS-PAGE. The gel was blotted onto a nitrocellulose membrane (GE Healthcare) and probed with mouse monoclonal anti-CKB (B-9; 1:1000; Santa Cruz Biotechnology) and mouse monoclonal anti-S100A4 (A-7; 1:1000; Santa Cruz Biotechnology). Goat anti-mouse IgG horseradish peroxidase-conjugated antibodies (1:5000; BioRad) were used as secondary antibodies. Detection was via chemiluminescence procedures (Pierce). Ponceau S staining was used as loading control [27].

2.6. Data analysis

Gene Ontology (GO) annotations were analyzed using the PHANTER overrepresentation test (release 20150430) [28] with the GO ontology database released 2015-08-06. Student's *t* test was used to analyze differences in fold change, amount of protein and relative migration. Differences with *P* values of 0.05 or less were considered significant. Other analyses are described in Supplementary materials and methods.

2.7. Cell adhesion and cell migration assays

These assays were carried out as previously described [26].

3. Results and discussion

3.1. Analysis of HeLa cells transfected with slrP by two-dimensional protein gels

We previously generated a stable *slrP-3xFLAG*-transfected HeLa cell line that was useful to show a role of the *Salmonella* effector SlrP in interfering with host thioredoxin activity and in contributing to the induction of host cell death [20]. The same cells were used in this study to explore the global effect of SlrP on the proteome of human epithelial cells. Protein extracts were prepared from these cells and from a control cell line that was transfected with the empty vector. Proteins were separated using two-dimensional gel electrophoresis and differentially expressed products were isolated. Fig. 1 shows representative images of control and SlrP-expressing cells with relevant spots marked. From 23 initially selected proteins, 16 were unambiguously identified by mass spectrometric analysis (Table 1). All of them were down-regulated in the presence of SlrP.

3.2. Analysis of slrP transfected cells versus control cells using iTRAQ

The technique used in the previous section has some intrinsic limitations linked to the chemical diversity of proteins and their very divergent expression in cells [29]. In order to increase our ability to identify proteins whose expression was affected by SlrP, extracts from the same cells (two extracts from *slrP*-transfected and two extracts from control cells) were also analyzed using a quantitative proteomics iTRAQ method [30]. A total of 505 proteins were identified (Table S1). Among them, 27 were differentially expressed in the presence of SlrP according to the criteria of reaching a ratio statistically different from 1 ($P < 0.05$) and higher than 1.3- (11 proteins) or lower than 0.7-fold (16 proteins) (Table 2). A comparison between the results of the two techniques, two-dimensional

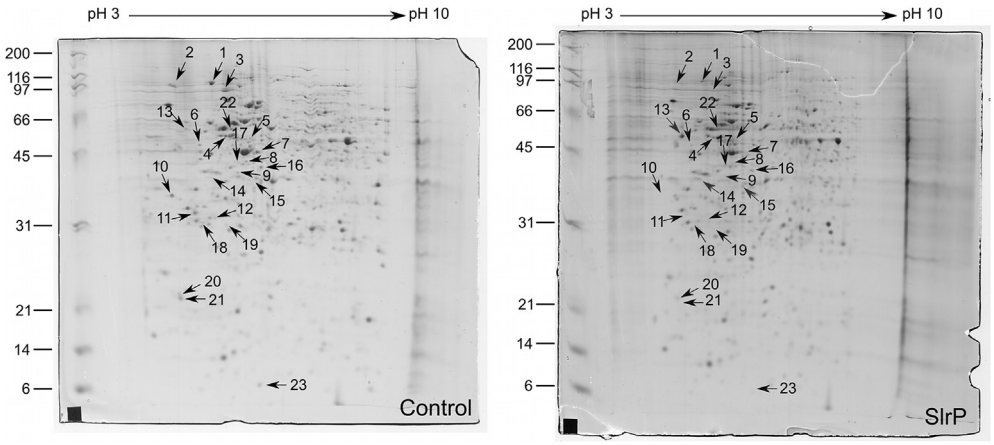

Fig. 1. Effects of SlrP on the proteome of HeLa cells. Protein extracts from HeLa cells stably transfected with pcDNA3 (control) or with pcDNA3-SlrP-3xFLAG (SlrP) were analyzed by two-dimensional gel electrophoresis. Numbered arrows indicate species differentially expressed in the presence of SlrP.

Table 1
Differentially expressed proteins in HeLa cells expressing SlrP identified in two-dimensional gel electrophoresis and comparison with mRNA ratios.

Spot[a]	Gene symbol	Protein name	Protein score[b]	Peptide count	mRNA ratio	*P* value (mRNA)
1	HSP90B1	Endoplasmin	824	28	–	–
2	PRKCSH	Glucosidase 2 subunit beta	267	14	–	–
3	HSP90AB1	Heat shock protein HSP 90-beta	681	32	–	–
6	RNH1	Ribonuclease inhibitor	792	24	1.02 ± 0.08	0.7707
7	CKB	Creatine kinase B-type	876	23	0.96 ± 0.07	0.4019
8	SERPINB6	Serpin B6	625	21	0.82 ± 0.01	0.0012
9	SEC13	Protein SEC13	281	9	1.01 ± 0.11	0.9232
10	C1QBP	Complement component 1 Q subcomponent-binding protein	63	5	1.08 ± 0.03	0.1222
11	YWHAE	14-3-3 protein epsilon	469	22	1.12 ± 0.11	0.0744
12	YWHAG	14-3-3 protein gamma	186	11	0.92 ± 0.07	0.2157
13	BASP1	Brain acid soluble protein	257	7	0.97 ± 0.02	0.3910
16	EIF3I	Eukaryotic translation initiation factor 3 subunit I	588	17	1.02 ± 0.06	0.6579
17	HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	157	12	–	–
19	CAPNS1	Calpain small subunit 1	248	12	1.08 ± 0.04	0.1509
22	TUBB4B	Tubulin beta-4B chain	634	25	0.99 ± 0.08	0.9231
23	S100A4	Protein S100-A4	217	8	0.75 ± 0.05	0.0007

[a] Numbers as indicated in Fig. 1. All the identified spots were decreased in the presence of SlrP.
[b] According to MS + MS/MS analysis.

gel electrophoresis and iTRAQ, reveals that: (i) Differential expression of two proteins, CKB and S100A4, was confirmed by both methods. (ii) There were conflicting results, regarding the proteins HNRNPC, HSP90AB1, HSP90B1 and PRKCSH, identified as decreased in the presence of SlrP using the two-dimensional gels but whose expression was not altered according to the iTRAQ approach. The reasons are unknown, but these proteins were not included in the analysis below. (iii) No data were obtained with iTRAQ for 10 additional proteins identified with the first technique. In summary, both approaches can be seen as complementary, since a fraction of the total proteome was analyzed with each technique and the overlapping between them was only partial.

3.3. Comparison between mRNA and protein levels for differentially expressed proteins

To investigate if the changes in protein expression correlated with changes in the corresponding mRNA, we carried out a genome-wide analysis of transcripts from *slrP*-transfected cells compared to control cells using Affymetrix Human Gene 1.0 ST Arrays. The complete results of this analysis will be reported elsewhere, but for this study we extracted the data obtained for the genes whose products are differentially expressed according to the proteomic techniques. Data shown in Tables 1 and 2 indicate that there was a good correlation between mRNA and protein changes for 16 out of 37 genes. SlrP has been shown to be located mainly in the cytosol of the host cell and partially in the endoplasmic reticulum [20,23]. It is not expected to translocate into the nucleus and directly control transcription. However, its presence in the host cell has clearly an impact that directly or indirectly could affect the activity of some transcriptional factors. We have previously shown a significant effect on transcription of a different set of genes caused by the expression of another *Salmonella* effector, SteA, in HeLa cells [26]. Interestingly, among the other 21 genes, 19 did not show significant changes of expression at the mRNA level and 2 were significantly decreased in the presence of SlrP at the protein level but increased at the mRNA level. These results suggest that SlrP can also affect expression of certain genes in a posttranscriptional manner. At least two direct mechanisms could explain these effects: (i) SlrP is able to physically interact with some proteins and modify their activities [20,23]. The interaction with other proteins could increase or decrease their stability. (ii) SlrP is an E3 ubiquitin ligase whose only known substrate is human Trx1 [20,22]. Although stability of Trx1 does not appear to be altered, ubiquitination of other substrates could lead to their proteasome-dependent degradation.

Table 2
Differentially expressed proteins in HeLa cells expressing SlrP quantified by iTRAQ and comparison with mRNA ratios.

Gene symbol	Protein name	Protein ratio	mRNA ratio	*P* value (mRNA)
ACAT1	Acetyl-CoA acetyltransferase, mitochondrial	0.52 ± 0.16	1.34 ± 0.16	0.0020
ANXA1	Annexin A1	0.68 ± 0.09	0.86 ± 0.05	0.0042
CKB	Creatine kinase B-type	0.59 ± 0.12	0.96 ± 0.07	0.4019
CRIP1	Cysteine-rich protein 1	0.69 ± 0.06	0.55 ± 0.06	2.711×10^{-5}
HPRT1	Hypoxanthine-guanine phosphoribosyltransferase	0.51 ± 0.12	0.69 ± 0.04	0.0001
KIAA0100	Protein KIAA0100	0.54 ± 0.05	1.13 ± 0.02	0.0119
KYNU	Kynunerinase	0.58 ± 0.17	0.28 ± 0.02	9.297×10^{-10}
LGALS1	Galectin-1	0.49 ± 0.09	0.65 ± 0.09	4.987×10^{-5}
PLAT	Tissue-type plasminogen activator	0.40 ± 0.03	0.87 ± 0.12	0.1100
PUS10	Putative tRNA pseudouridine synthase Pus10	0.68 ± 0.01	1.07 ± 0.03	0.1453
S100A4	Protein S100-A4	0.54 ± 0.08	0.76 ± 0.05	0.0007
ANXA6	Annexin A6	1.74 ± 0.32	1.45 ± 0.12	6.641×10^{-5}
CADPS2	Calcium-dependent secretion activator 2	2.21 ± 0.27	1.02 ± 0.17	0.8429
CAP1	Adenylyl cyclase-associated protein 1	1.66 ± 0.32	1.02 ± 0.05	0.6440
CMBL	Carboxymethylenebutenolidase homolog	1.97 ± 0.49	1.18 ± 0.07	0.0062
FASN	Fatty acid synthase	1.37 ± 0.23	1.81 ± 0.08	1.144×10^{-6}
FDPS	Farnesyl pyrophosphate synthase	1.37 ± 0.19	1.48 ± 0.11	0.0002
MYO16	Unconventional myosin-XVI	2.47 ± 0.35	0.94 ± 0.06	0.2709
NPEPPS	Puromycin-sensitive aminopeptidase	1.70 ± 0.20	0.95 ± 0.00	0.3751
PPA1	Inorganic pyrophosphatase	1.36 ± 0.04	1.18 ± 0.09	0.0216
RBBP7	Histone-binding protein RBBP7	1.35 ± 0.07	1.34 ± 0.01	3.146×10^{-5}
RBM39	RNA-binding protein 39	2.35 ± 0.43	1.12 ± 0.05	0.0282
RBMX	RNA-binding motif protein, X chromosome	1.88 ± 0.20	1.00 ± 0.07	0.9908
RPL18	60S ribosomal protein L18	1.37 ± 0.05	0.98 ± 0.02	0.5586
RPS3A	40S ribosomal protein S3a	1.43 ± 0.09	0.97 ± 0.21	0.5732
SPTBN1	Isoform 2 of spectrin beta chain, non-erythrocytic 1	1.33 ± 0.19	1.29 ± 0.01	0.0003
VDAC2	Voltage-dependent anion-selective channel protein 2	2.02 ± 0.51	1.33 ± 0.16	0.0004

3.4. Confirmation of the differential expression of CKB and S100A4 by Western blotting

To provide confirmation of differentially expressed proteins, we carried out Western blot analysis of CKB and S100A4, the two proteins that were detected by the two-dimensional gel analysis and the iTRAQ study. Fig. 2 shows a representative immunoblot image. Quantification indicated that both proteins were significantly downregulated in the presence of SlrP. This result is consisting with data obtained in two-dimensional gels and iTRAQ analysis and provides evidence that both techniques were reliable. Interestingly, creatine kinases, including brain-type creatine kinase (CKB), are a family of enzymes involved in energy homeostasis that reversibly catalyzes the transfer of phosphate between phosphorylated substrates as phosphocreatine and ATP. The dysfunction of these proteins has been linked with several types of disease and it has been reported their role as bacteriostatic factors capable of specifically inhibiting the growth of Gram negative bacteria [31]. Also, it was demonstrated the role of CKB in cell migration and phagocytosis by actin remodeling [32]. S100A4 is a member of the S100 calcium-binding protein family that is involved in many aspects of cell biology as cell motility, differentiation, cytoskeletal remodeling and survival [33]. Several members of this family are dramatically upregulated at the transcription level following different bacterial and viral infections, suggesting a role in the immune response [34]. In particular, S100A4 regulates bacterial clearance during staphylococcal infection [35]. It would be an intriguing possibility that SlrP, which is secreted by both T3SS, could modulate the expression of CKB and S100A4 according to the stage of infection.

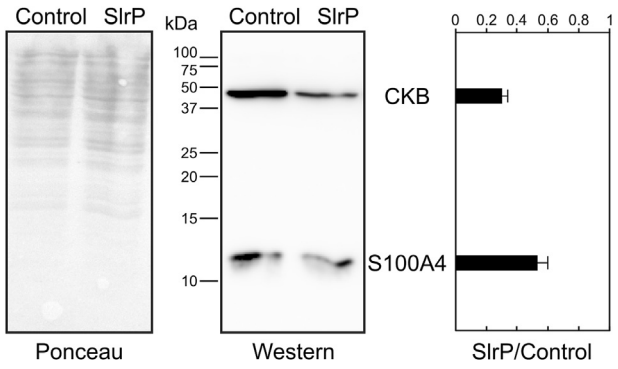

Fig. 2. Effects of SlrP on the expression of CKB and S100A4. Protein extracts from HeLa cells stably transfected with pcDNA3 (control) or with pcDNA3-SlrP-3xFLAG (SlrP) were analyzed by immunoblot with anti-CKB and anti-S100A4 antibodies. A representative gel (central panel) is shown together with quantification of bands from three gels (means + SD of CKB or S100A4/total protein ratio, right panel). Ponceau S staining (left panel) was used to estimate total loaded protein.

3.5. Gene ontology analysis and phenotypic analysis of transfected cells

Together, the two techniques used in this work provided 37 proteins (excluding those with conflicting results) that were differentially expressed in HeLa cells expressing SlrP. A gene ontology enrichment analysis was carried out on these proteins using the PANTHER overrepresentation test. Significant enrichment was detected for specific cellular components and molecular functions (Table 3). Interestingly, the ontology terms with the highest fold enrichment refer to several types of junctions mediating adhesion in epithelial cells, including focal adhesions and adherens junctions. Among the 7 proteins responsible for this enrichment, 3 are downregulated (annexin A1, 14-3-3 protein gamma, and 14-3-3 protein epsilon) and 4 are upregulated (annexin A6, adenylyl cyclase-associated protein 1, and ribosomal proteins S3a and L18) in the presence of SlrP. Adhesion structures are important to attach a cell to the extracellular matrix and to neighboring cells. In addition, focal adhesion turnover coordinated with actin dynamics has a crucial role in cell migration [36]. We hypothesized that the differential expression of proteins associated to adhesion complexes that is observed in *slrP*-transfected cells

Table 3
Gene ontology enrichment analysis of differentially expressed proteins in *slrP*-transfected cells.

GO category (p < 0.05) (top ten by fold enrichment)	Number of proteins	Expected	Fold enrichment
Cellular component			
focal adhesion (GO:0005925)	7	0.68	10.29
cell-substrate adherens junction (GO:0005924)	7	0.69	10.14
cell–substrate junction (GO:0030055)	7	0.70	10.00
adherens junction (GO:0005912)	7	0.82	8.54
anchoring junction (GO:0070161)	7	0.85	8.24
extracellular exosome (GO:0070062)	26	4.83	5.38
extracellular membrane-bounded organelle (GO:0065010)	26	4.83	5.38
extracellular organelle (GO:0043230)	26	4.85	5.36
extracellular vesicle (GO:1903561)	26	4.85	5.36
membrane-bounded vesicle (GO:0031988)	27	6.13	4.41
Molecular function			
RNA binding (GO:0003723)	18	2.77	6.50
poly(A) RNA binding (GO:0044822)	11	2.05	5.37
nucleic acid binding (GO:0003676)	20	7.00	2.86
heterocyclic compound binding (GO:1901363)	26	10.36	2.51
organic cyclic compound binding (GO:0097159)	26	10.49	2.48
binding (GO:0005488)	36	24.81	1.45

could result in defects in adhesion and/or migration. Indeed, when these cells were cultured in poly-HEMA coated tissue culture plates (to prevent adhesion to the substrate), they were loosely dispersed in the media whereas control cells formed dense aggregates, indicating a defect in cell–cell adhesion in cells expressing SlrP (Fig. 3A). A wound closure assay also showed that migration speed was altered in these cells (Fig. 3B). Together, these data indicate a good correlation between the results of the proteomic analysis and the phenotypic consequences of SlrP expression.

The analysis of the function of individual T3SS effectors is obscured by the fact that many effectors are translocated into the host cell during an infection, sometimes with redundant or opposite results. The strategy of expressing a particular effector in host cells circumvents this problem. Here, we used a proteomic approach to study the effect of the stable expression of the effector SlrP on HeLa cells. The results obtained with this approach may be useful to guide future research on the role of this *Salmonella* effector. In fact, we were able to show differential expression of 37 genes at the protein level and, interestingly, most of these genes are not altered at the mRNA level. Since SlrP is a ubiquitin ligase [20,22] that physically interacts with at least two host proteins [20,23], it would be interesting to study if SlrP is able to posttranscriptionally modify the expression of those genes through direct interaction with and/or ubiquitination of some of their products.

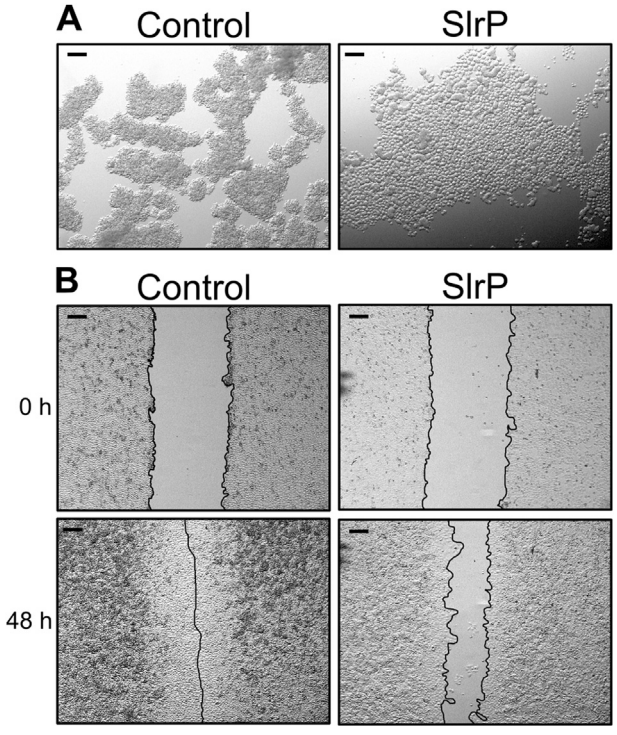

Fig. 3. Defects in cell–cell adhesion and cell migration in HeLa cells expressing SlrP. (A) HeLa cells were transferred to poly-HEMA-coated plates. After 48 h of culture in suspension, control cells HeLa pcDNA3 (control) and HeLa pcDNA3-SlrP-3xFLAG (SlrP) were photographed under an inverted microscope. (B) Representative images of wound-healing assays for HeLa pcDNA3 (control) and HeLa pcDNA3-SlrP-3xFLAG (SlrP). Wounds are outlined. Scale bars, 200 μm.

Acknowledgments

We are grateful to Elena Cardenal-Muñoz (University of Geneva) for assistance with cell adhesion and migration experiments. This work was supported by the Spanish Ministry of Science and Innovation and the European Regional Development Fund (grants SAF2010-15015 and SAF2013-46229-R), and Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía, Spain (grant P08-CVI-03487).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.03.014.

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