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Respiratory syncytial virus (RSV) suppression of glucocorticoid receptor phosphorylation does not account for repression of transactivation ☆

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ABSTRACT

Respiratory syncytial virus (RSV)-induced bronchiolitis in infants, although inflammatory in nature, is not responsive to glucocorticoids. We have recently shown that RSV-infected lung epithelial cells have impaired glucocorticoid receptor (GR)-mediated transactivation. In this study, we show that the N-terminal region of GR is required for RSV repression of GR transactivation and that RSV infection of lung epithelial cells reduces ligand-dependent GR phosphorylation at serine 211 and serine 226. However, we also show that these changes in GR phosphorylation do not account for the RSV repression of GR transactivation suggesting other regions of the GR N-terminus must also be involved.

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1. Introduction

Respiratory syncytial virus (RSV) is the major cause of severe lower respiratory tract infections in children, resulting in 132,000–172,000 infant hospitalizations/year in the USA alone [1]. Treatment of severe RSV symptoms in children is estimated to cost $600 billion per year in the USA [2]. In addition to children, immunocompromised adults and the elderly are also at risk from severe RSV disease [3,4].

RSV is a negative-sense single-stranded RNA pneumovirus of the *Paramyxoviridae* family that causes bronchiolitis, an inflammatory disease of the bronchioles. Glucocorticoids, one of the most powerful anti-inflammatory agents available, have no beneficial effect for infants with RSV-induced bronchiolitis [5–8]. In addition, glucocorticoids show impaired suppression of RSV-induced cytokines in *in vitro* assays [9–11]. These data, suggest that RSV may have a deleterious effect on glucocorticoid signaling. In fact, we have recently shown that RSV infection represses glucocorticoid receptor (GR)-mediated transactivation [11].

Glucocorticoids play an important role in regulating many systems including the immune system. They function through GR, a cytosolic receptor. Upon ligand binding, GR dissociates from a protein complex, dimerizes and translocates to the nucleus where it binds to specific DNA sequences, glucocorticoid response elements (GREs), to activate gene transcription. GR consists of three functional domains – N-terminal transactivation domain, central DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD). The ability to activate gene transcription is dependent on two activation domains, AF1 and AF2, located in the N-terminus and C-terminal LBD, respectively. In addition, transcriptional activity can also be modulated by phosphorylation [12,13].

We have previously shown that RSV infection impairs GR-mediated transactivation by interfering with GR binding to the GREs in the promoters of responsive genes [11]. In this study we show that RSV infection also results in reduced GR phosphorylation, but changes in phosphorylation alone cannot account for the repression of GR-mediated transactivation observed in RSV-infected lung epithelial cells.

2. Materials and methods

2.1. Materials

Dexamethasone was purchased from Sigma–Aldrich (St. Louis, MO) and was dissolved in 99.5% ethanol. Calyculin A was purchased from Cell Signaling Technology, Inc. (Danvers, MA) and okadaic acid from EMD Millipore (Billerica, MA). Both were dissolved in DMSO.

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2.2. Cell culture

A549 cells, an alveolar cell carcinoma derived cell line which retains features of the type II alveolar epithelial cells, is routinely used as a model for RSV infection of epithelial cells [14]. A549 cells (American Type Culture Collection (ATCC), Manassas, VA) were grown in DMEM/F12 (50/50) media with 10% fetal bovine serum (FBS) and Cos7 cells (ATCC), were grown in DMEM media with 10% FBS at 37 °C and 5% CO_2.

2.3. Virus preparation and infection

Recombinant green fluorescent protein (GFP)-expressing RSV (rgRSV) was grown in HeLa cells, separated from debris by low speed centrifugation and further purified by pelleting in a high speed centrifuge [11,15]. Viral adsorption was performed as previously described [11]. Successful infection was confirmed by the presence of GFP viewed under a fluorescent microscope.

2.4. Transient transfection

Cos7 cells were plated in 24-well cell culture plates in DMEM containing 10% CSS at a concentration of 5×10^4 cells/well. The next day cells were transfected with 20 ng GR, 100 ng of the GR-responsive promoter, MMTVLuc or the GAL-4-responsive promoter, FRLuc promoter (Agilent Technologies, Santa Clara, CA), 20 ng of the renilla internal control, pRL TK (Promega Corp., Madison, WI) and 360 ng pSG5 using TransIT-LT1 (Mirus Bio, Madison, WI) according to the manufacturer's instructions. The following day the cells were inoculated with rgRSV (multiplicity of infection (MOI) = 3). After 1 h the inoculum was replaced with media containing vehicle or 100 nM dexamethasone. After 5 or 24 h the cells were lysed and luciferase measured using the Dual Luciferase Assay (Promega Corp.) on a Packard LumiCount luminometer (PerkinElmer, Waltham, MA).

2.5. Western blotting

Total cellular protein was isolated using M-PER in the presence of protease and phosphatase inhibitors (Thermo Fisher Scientific Inc., Pittsburgh, PA) according to the manufacturer's instructions. 10 μg protein was subjected to SDS–PAGE on NuPAGE Novex 4–12% bis–tris pre-cast gels (Life Technologies, Grand Island, NY). Proteins were transferred to nitrocellulose membranes by semi-dry blotting and blocked in 5% non-fat dry milk in Tris-buffered saline–0.05% Tween 20 (TBST). They were incubated with primary antibody in 5% milk in TBST overnight at 4 °C. Membranes were washed three times with TBST and incubated with secondary antibody in TBST for 1 h at room temperature. Membranes were again washed with TBST and chemiluminescence detected using SuperSignal West Pico Chemiluminscence Substrate (Thermo Fisher Scientific Inc.) according to the manufacturer's instructions and exposed to autoradiographic film or visualized using a Kodak Image Station 4000MM Pro (Carestream Health Inc., Rochester, NY). Membranes were stripped for 15 min at room temperature in Restore Stripping buffer (Thermo Fisher Scientific Inc.) and re-probed as needed. Blots were quantified using ImageJ software (NIH). Primary antibodies used were against phospho-GR (Ser211) (Cell Signaling Technology Inc.; 4161; 1:1000), phospho-GR (Ser226) (Abcam, Cambridge, MA; ab53692; 1:500), GR (H-300) (Santa Cruz Biotechnology, Santa Cruz, CA; sc-8992; 1:1000), RSV (EMD Millipore; AB1128; 1:5000) and β-actin (Santa Cruz Biotechnology; sc-47778; 1:2000). The secondary antibodies used were goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology; sc-2004; 1:2000), bovine anti-goat IgG-HRP (Santa Cruz Biotechnology; sc-2350; 1:2000) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology; sc-2005; 1:5000).

2.6. Real-time PCR

Total RNA was extracted using TRIzol (Life technologies) according to the manufacturer's instructions. 500 ng RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with RNase inhibitor (Life Technologies) in a 20 μl reaction. cDNA was then diluted by addition of 100 μl DEPC-treated H_2O. Real-time PCR was performed on 8 μl diluted cDNA in a 20 μl reaction containing $1 \times$ Power SYBR® Green PCR Master Mix (Life technologies) and 0.125 μM of each primer ([11] and Table 1) using the following protocol on an ABI 7300 Real-Time PCR system. Plates were heated at 50 °C from 2 min, denatured for 10 min at 95 °C and subjected to 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Cycle threshold (C_T) values of duplicate samples were analyzed using the comparative CT ($\Delta\Delta C_T$) method (Life technologies). The fold induction ($2^{-\Delta\Delta Ct}$) by dexamethasone was obtained by normalizing to two endogenous genes, GAPDH (glyceraldehyde-3-phosphate dehydrogenase) and CAP-1 (Cyclic AMP-accessory protein) [16], and expressed relative to the amount in non-treated cells. Relative copy numbers (RCN) were calculated as $2^{-\Delta Ct} \times 100$ [16].

2.7. siRNA-mediated gene silencing

50 nM protein phosphatase 5 (PP5), pan protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A-Cα) siRNA and control siRNA-A (Santa Cruz Biotechnology; sc-44602, sc-43545, sc-43509 and sc-37007, respectively) was transfected into A549 cells using DharmaFECT 2 (Thermo Fisher Scientific Inc.,) according to the manufacturer's instructions. 48 h post transfection the cells were inoculated with rgRSV (MOI = 3). After 1 h the inoculum was removed and cells treated with vehicle or 100 nM dexamethasone and the expression glucocorticoid-inducible leucine zipper (GILZ) determined by real-time PCR. The efficiency of siRNA silencing was determined by real-time PCR.

2.8. Cloning of pCMV HA-hGR S211A S226A double mutant

The double mutant pCMV HA-hGR S211A S226A was generated by insertion of the S226A mutation into the pCMV HA-hGR S211A plasmid using the QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies) according to manufacturer's instructions. Successful mutagenesis was confirmed by sequencing.

2.9. Statistical analysis

Transfection experiments were performed in triplicate and real-time experiments in duplicate. The average values of n independent experiments were analyzed for statistical significance using the two-tailed unpaired Student's t-test using Prism 5 software (* depicts $p = 0.05–0.01$; ** depicts $p = 0.01–0.001$; *** depicts $p < 0.001$).

3. Results

3.1. N-terminal region of GR is required for RSV repression of GR-mediated transactivation

To define which region of GR was needed for the RSV repression of GR-mediated transactivation, expression plasmids for wild type rat GR (rGR) and the N-terminal deletions – pSG5/GR407C, that lacks the N-terminal 407 amino acids of GR, but retains the DBD and LBD regions [17], and GAL/GR525C, that lacks the N-terminal 525 amino acids of GR, but retains purely the LBD attached to the gal 4 DBD [18], were used. In the absence of transfected GR, dexamethasone did not induce the MMTVLuc promoter, confirming that Cos7 cells contain very little, if any, of their own GR. Dexamethasone induced a 48-fold

Table 1
Real-time PCR primers.

Gene name	Accession number	Direction	Primer sequence (5′-3′)	Reference
CAP-1	NM_006367.3	F	ATTCCCTGGATTGTGAAATAGTC	[16]
		R	ATTAAAGTCACCGCCTTCTGTAG	
GAPDH	NM_002046.3	F	ACTTTGGTATCGTGGAAGGACT	[16]
		R	GTAGAGGCAGGGATGATGTTCT	
GILZ	NM_004089	F	GCACAATTTCTCCATCTCCTTCTT	[11]
		R	TCAGCATGATTCTTCACCAGATCCA	
PPP5C	NM_006247.3	F	GCTGGACATCGAGAGCATGACCATT	
		R	ACGAGCGTGCTCAGCTTGGA	
PPP1CA	NM_002708.3	F	CCTGCTGGAAGTGCAGGG	
		R	GAAGGTCGTAGTACTGGCCG	
PPP2CA	NM_002715.2	F	GCCTCTGCGAGAAGGCTAAA	
		R	CCCATGCACATCTCCACAGA	
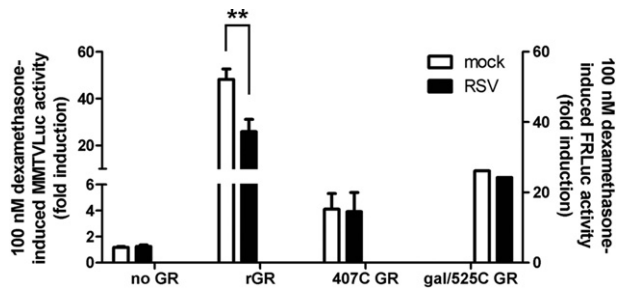
Fig. 1. GR N-terminal region is required for RSV-mediated repression of dexamethasone-induced promoter activity. Cos7 cells were transfected with full length rGR, pSG5/GR407C, or GAL/GR525C; MMTVLuc or the FRLuc promoter; and a control renilla vector. They were RSV (MOI = 3)- (black) or mock-infected (white) and then treated with dexamethasone for 5 h. Firefly luciferase was normalized to renilla and fold increase following dexamethasone treatment calculated. Means and SD are shown ($n = 3$).

Fig. 2. A protein phosphatase inhibitor reverses RSV-induced repression of dexamethasone-induced GR phosphorylation. A549 cells were mock- (lanes 1–3) or RSV (MOI = 3)-infected (lanes 4–5) and then at 5 h.p.i. treated with vehicle (lane 1) or 100 nM dexamethasone (lanes 2–5) for 30 min in the presence (lanes 3 and 5) or absence of 10 nM calyculin A. Similarly, cells were mock- (lanes 6–8) or RSV (MOI = 1)-infected (lanes 9–11) and then at 24 h.p.i. treated with vehicle (lanes 6 and 9) or 100 nM dexamethasone (lanes 7–8 and 10–11) for 30 min in the presence (lanes 8 and 11) or absence of 10 nM calyculin A. Whole cell lysates were prepared and 10 μg protein subjected to SDS–PAGE and western blotting using antibodies against phospho-GR (Ser211), phospho-GR (Ser226), total GR, RSV and β-actin (A). Total GR was normalized to β-actin for cells infected at an MOI = 3 (B) and MOI = 1 (C). Phospho GR (Ser 211) was normalized to total GR for cells infected at an MOI = 3 (D) and MOI = 1 (E). Phospho GR (Ser 226) was normalized to total GR for cells infected at an MOI = 3 (F) and MOI = 1 (G). A representative figure of 3 independent experiments is shown.
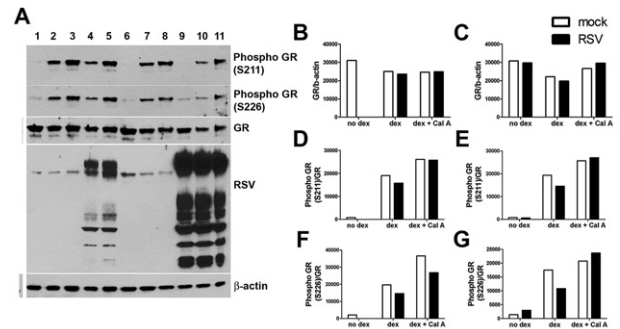
increase in the MMTVLuc promoter activity in mock-infected, wild type rGR transfected cells. RSV infection significantly reduced the induction of the MMTVLuc promoter to 26-fold. Dexamethasone induced a 5.6-fold increase in the MMTVLuc promoter activity in mock-infected, pSG5/GR407C transfected cells that was not repressed by RSV infection. Finally, dexamethasone induced a 26-fold increase in the FRLuc promoter in mock-infected, GAL/GR525C transfected cells that was not repressed by RSV infection (Fig. 1).

3.2. Effect of RSV infection on GR phosphorylation

Within the N-terminal region of GR are phosphorylation sites that are associated with transcriptional activity [12,13]. Dexamethasone induces GR phosphorylation at serine 211 which is suppressed by RSV infection. This suppression of dexamethasone-induced GR phosphorylation at serine 211 is seen at 5 hours post infection (h.p.i.) in RSV (MOI = 3)-infected A549 cells (Fig. 2A, lanes 2 and 4, Fig. 2D) and at 24 h.p.i. in RSV (MOI = 1)-infected cells (Fig. 2A, lanes 7 and 10, Fig. 2E). Similar results were seen for dexamethasone-induced phosphorylation of GR at serine 226 (Fig. 2A, lanes 2 and 4, 7 and 10, Fig. 2F and G). No change in total GR levels was observed (Fig. 2A–C) as reported previously [11].

3.3. Role of protein phosphatases in RSV repression of GR function

We first tested if inhibition of protein phosphatases could reverse RSV effects on GR phosphorylation. The protein phosphatase inhibitor calyculin A increased dexamethasone-induced GR phosphorylation at both the serine 211 and serine 226 sites such that there was now no difference between the mock- and RSV-infected cells (Fig. 2D, E, and G), except for serine 226 at an MOI of 3 (Fig. 2F).

We next tested if inhibition of protein phosphatases could reverse RSV suppression of GR function. Dexamethasone induction of the glucocorticoid-inducible gene, GILZ was repressed 76% by RSV infection in the absence of protein phosphatase inhibitors. Pre-treatment with okadaic acid reduced this slightly, but not significantly, to 60% (Fig. 3A). Likewise, pre-treatment with calyculin A also reduced this slightly, but not significantly, to 59% (Fig. 3B).
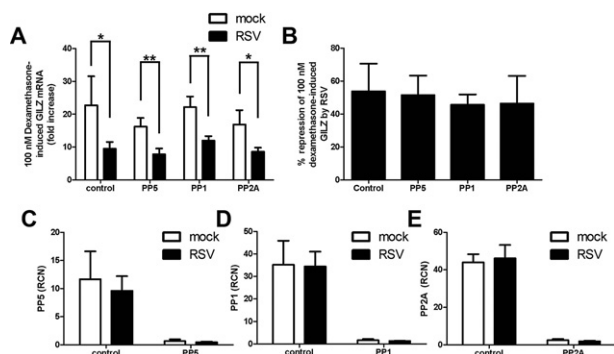
We next tested if knock-down of protein phosphatases was able to reverse the effects of RSV infection on GR-mediated transactivation. RSV infection significantly reduced dexamethasone induction of GILZ in the absence and presence of all siRNAs (Fig. 4A). The RSV-mediated % repression of dexamethasone-induced GILZ was not altered by the presence of PP5, PP1 and PP2A siRNA (Fig. 4B). siRNA knock-down was confirmed by real-time PCR (Fig. 4C–E).

3.4. Effect of RSV infection on GR phospho mutants

To fully determine if the changes in GR phosphorylation are required for RSV repression of GR-induced gene activation, we used plasmids expressing single serine to alanine point mutations in one of the three GR phosphorylation sites: serine 211, 226 and 203 [13,19]. As we have seen changes in both serine 211 and 226 phosphorylation
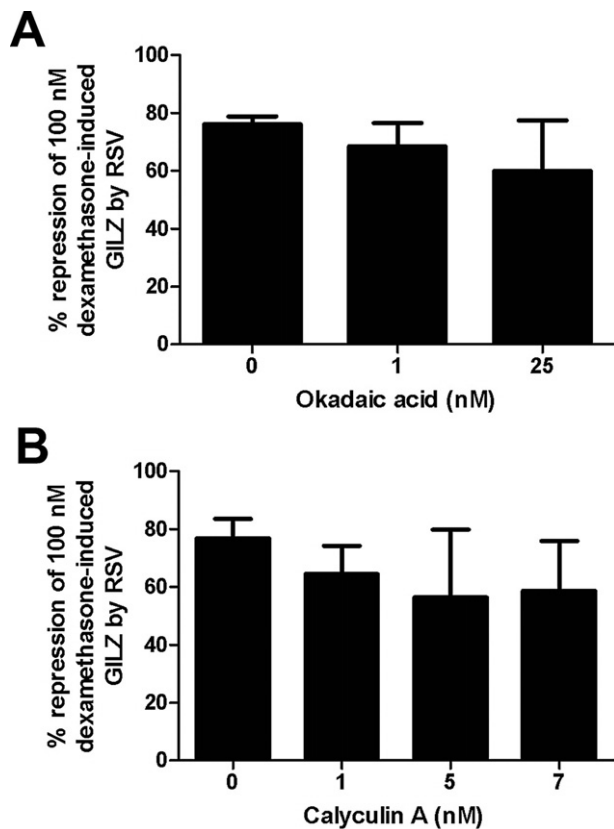
Fig. 3. Protein phosphatase inhibitors do not reverse RSV repression of dexamethasone-induced GILZ. A549 cells were RSV (MOI = 3)- or mock-infected and treated with okadaic acid (A) or calyculin A (B) for 30 min followed by treatment with vehicle or 100 nM dexamethasone for 5 h. Dexamethasone-induced GILZ mRNA was determined by real-time PCR and normalized to GAPDH and CAP-1. % repression by RSV was calculated as a percentage of ((fold increase mock − fold increase RSV)/fold increase mock). Means and SD are shown (n = 3 (A) or 4 (B)).

Fig. 4. Knock-down of protein phosphatases does not reverse RSV repression of dexamethasone-induced GILZ. A549 cells were transfected with siRNA against PP5, PP1 and PP2A or a control siRNA. 48 h later they were RSV (MOI = 3)- (black) or mock-infected (white) and treated with vehicle or 100 nM dexamethasone for 5 h. Dexamethasone-induced GILZ mRNA was determined by real-time PCR and normalized to GAPDH and CAP-1 (A). % repression by RSV was calculated as previously (B). siRNA knockdown was confirmed by real-time PCR (C–E). Means and SD are shown (n = 4).

in response to RSV infection (Fig. 2), we generated the double mutation S211A S226A. All these constructs induced MMTVLuc activity in a ligand dependent manner, which was significantly repressed by RSV infection (Fig. 5).
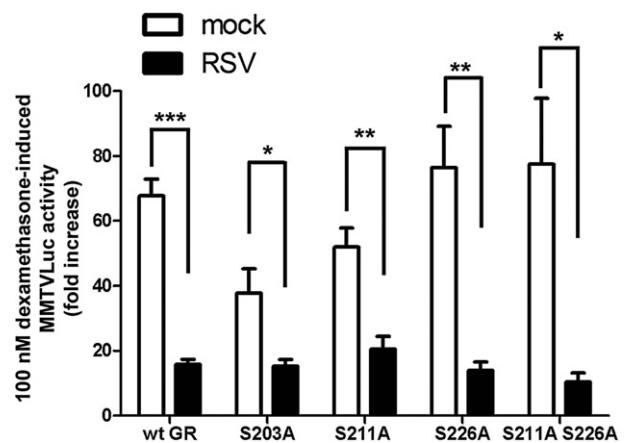
Fig. 5. GR phosphorylation point mutations do not reverse RSV repression of dexamethasone-induced MMTVLuc activity. Cos7 cells were transfected with full length pCMV HA-hGR, pCMV HA-hGR S203A, pCMV HA-hGR S211A, pCMV HA-hGR S226A or pCMV HA-hGR S211A S226A; MMTVLuc promoter; and a control renilla vector. They were RSV (MOI = 3)- (black) or mock-infected (white) and treated with 100 nM dexamethasone for 24 h. Firefly luciferase was normalized to renilla and fold increase following dexamethasone treatment calculated. Means and SD are shown (n = 4).

4. Discussion

We have previously shown that RSV infection of lung epithelial cells interferes with glucocorticoid-induced gene activation through inhibition of GR-DNA binding [11]. To further investigate the mechanism by which this repression occurs we sought to identify which region of GR was necessary for the repression of GR-mediated transactivation by RSV. Here we have shown that the N-terminal region of GR is required for RSV repression of GR-mediated transactivation (Fig. 1). This region contains AF1, a transactivation domain, and a number of GR phosphorylation sites.

Following ligand treatment, human GR is phosphorylated at serine 211, 226 and 203 [13]. Phosphorylation of serine 211 results in GR nuclear localization, DNA binding, and correlates with transcriptional activity. Phosphorylation of serine 226 also results in nuclear localization and DNA binding, whereas phosphorylation of serine 203 inhibits nuclear localization [12,13]. Given the association of phosphorylation of serine 211 and 226 with transcriptional activity, we tested the effect of RSV infection on the ligand-dependent phosphorylation of these sites. RSV infection of A549 cells inhibited ligand dependent phosphorylation of both serine 211 and 226 (Fig. 2).

Changes in phosphorylation state may be the result of changes in kinase or phosphatase activity. Treatment with the protein phosphatase inhibitor calyculin A increased GR phosphorylation in the absence of RSV-infection suggesting inhibition of endogenous protein phosphatase activity that limits ligand-dependent GR phosphorylation. In addition, calyculin A also reversed the effects of RSV on ligand dependent phosphorylation (Fig. 2), such that GR phosphorylation levels were similar in mock- and RSV-infected cells, suggesting a role for enhanced protein phosphatase activity in the RSV effects on GR. Protein phosphatases have previously been implicated in GR function. Okadaic acid, a protein phosphatase inhibitor, increases GR-DNA binding and nuclear accumulation [20]. In addition, PP5 is associated with the C-terminal LBD of GR [21] and dephosphorylates GR at serine 211 [22]. Suppression of PP5 increases GR nuclear accumulation, DNA binding, and transcriptional activity [22–24]. In addition, PP5 has been shown to play a role in cytokine suppression of GR transactivation [25]. Although much research has focused on PP5, other protein phosphatases may also play a role in regulation of GR phosphorylation. For example, PP2A has been reported to play a role in glucocorticoid insensitivity in asthma [26]. In order to determine if these

changes in GR phosphorylation are required for the RSV repression of GR transactivation, we tested if the protein phosphatase inhibitors okadaic acid or calyculin A could reverse the effects of RSV infection on dexamethasone induction of GILZ. We found only a slight, non-significant, reduction in RSV repression of dexamethasone-induced GILZ mRNA (Fig. 3). In addition, we knocked-down the expression of PP5, PP1 and PP2A and similarly found only a slight, non-significant, reduction in RSV repression of dexamethasone-induced GILZ mRNA (Fig. 4). It should be noted that we only used individual siRNAs and there may be some redundancy in these protein phosphatases or with other protein phosphatases. However, from this data we cannot conclude that the RSV changes in GR phosphorylation play a role in RSV repression of GR transactivation.

Finally, we tested the effect of RSV on GR phospho point mutations. Due to the repression of both serine 211 and 226 phosphorylation by RSV and the fact that some interplay between these phosphorylation sites has been reported [19,27], we also included the double mutant S211A S226A. RSV infection repressed dexamethasone-induced transactivation through all of the phospho point mutated GRs as well as wild-type GR (Fig. 5). These data suggest that RSV mediated changes in GR phosphorylation alone cannot account for RSV repression of GR transactivation and that other regions of the N-terminus of GR must be required. Further studies are needed to investigate which other regions of the N-terminus are required and if these other regions work in collaboration with these changes in phosphorylation.

Acknowledgements

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