Glucocorticoid Receptor Interacting Protein-1 restores glucocorticoid responsiveness in steroid-resistant airway structural cells.

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● **Running title:** GRIP-1 upregulation overcomes steroid resistance

ABSTRACT

Glucocorticoid (GC) insensitivity presents a profound management challenge in patients with asthma since conventional therapies are ineffective. The mutual inhibition exerted by the transcription factors and the GC receptor (GR) represents a possible mechanism contributing to GC insensitivity in asthma. We recently reported that Interferon Regulatory Factor (IRF)-1 is a novel transcription factor that promotes GC insensitivity in human airway smooth muscle (ASM) cells by interfering with GR signaling **(Tliba et al., 2008; Am J Respir Cell Mol Biol. 2008 Apr;38(4):463-72)**). We sought to determine whether the inhibition of GR function by IRF-1 involves its interaction with the transcriptional co-regulator GR-interacting protein 1 (GRIP-1), a known GR transcriptional co-activator. We here found that siRNA-mediated GRIP-1 depletion attenuated IRF-1-dependent gene transcription in cells transfected with a luciferase reporter construct. Similarly siRNA GRIP-1 dramatically decreases the mRNA expression of a IRF-1-dependent gene, CD38. In parallel experiments, we also found that silencing GRIP-1 significantly reduced GR-mediated transactivation activities. Immunoprecipitation and GST pull-down assays showed that GRIP-1, through its repression domain, physically interacts with IRF-1identifying GRIP-1 as an essential transcriptional co-activator for IRF-1. Interestingly, the previously reported inhibition of GR-mediated transactivation activities by either TNFα and IFNγ treatment or IRF-1 over-expression was fully reversed by increasing cellular levels of GRIP-1. Together, these data suggest that the cellular accumulation IRF-1 may represent a potential molecular mechanism mediating altered cellular response to GC through the depletion of GRIP-1 from the GR transcriptional regulatory complexes.

Key words: steroid insensitivity, asthma, glucocorticoid, cytokine, mesenchymal cells, airway smooth muscle, transcription factor, remodeling, IRF-1, GRIP-1, GR.

Glucocorticoids (GCs) remain the cornerstone treatment for chronic inflammatory diseases such as asthma (1). Five to ten percent of the asthmatic patients, however, develop steroid insensitivity. Therefore, an unmet need requires the exploration of alternative therapeutic targets to be used in conjunction with or separately from GCs (2). A strong correlation between steroid insensitivity and inflammatory diseases, such as asthma, nasal polyps and inflammatory bowel disease (3-5), prompted investigators to examine whether inflammatory mediators modulate the cellular responses to steroids. Using peripheral blood mononuclear cells (PBMCs), investigators showed that GC effects were dramatically reduced in the presence of cytokines. Leung's group was the first to show that IL-2 and IL-4 reduced the inhibitory effect of methylprednisolone on mitogen-induced T cell proliferation (6). Other cytokines such as IL-1β, IL-6, IFNγ to IL-2, IL-7, IL-13, IL-15 or IL-8 also attenuate dexamethasone effects in PBMCs (7-10), proliferating T cells, monocytes and neutrophils. Thus, cytokines may promote the development of steroid insensitivity seen in patients with asthma by reducing cell/tissue sensitivity to GCs.

Despite considerable efforts in immune cells, the alteration of steroid responsiveness in structural cell types of the target tissues remains poorly defined. We have been studying the modulation of steroid responsiveness in airway smooth muscle (ASM) which is increasingly recognized as an important player in the pathogenesis of asthma by driving airway inflammation (11) and may therefore be a target for inhaled GCs (12). Accordingly, we and others showed that GCs were effective in abrogating the expression of a number of pro-inflammatory cytokines, chemokines and adhesion molecules in ASM cells when exposed to a "single" pro-inflammatory stimulus (13). Yet, we recently found that steroid insensitivity can develop in ASM exposed to a

70 “mixture” of pro-asthmatic cytokines. Treatment of ASM cells with the specific combination of
71 IFNs with TNFα impairs the ability of GCs to inhibit the expression of calcium regulatory
72 protein CD38, the chemokines RANTES and fractalkine, and cell surface proteins such as
73 ICAM-1 and Toll-like receptor-2 (TLR2) (8).

74 The mechanisms underlying cytokine-induced steroid insensitivity in ASM cells have not
75 been completely defined but we recently showed that a “short-term” treatment of ASM cells with
76 IFNs and TNFα partially inhibits steroid transactivation through the accumulation of interferon
77 regulatory factor (IRF)-1 (14), an early response gene involved in diverse transcriptional
78 regulatory processes (15). Interestingly, polymorphism in IRF-1 has been associated with
79 childhood atopic asthma (16). Because expression of IRF-1 is increased after viral infections (17)
80 and because IRF-1 suppresses steroid effectiveness in ASM cells (14), we proposed that IRF-1
81 may mediate the reduced steroid responsiveness seen in patients with asthma experiencing viral
82 infections (18).

83 The precise transcriptional mechanism by which IRF-1 interferes with GC signaling
84 remains to be determined. Most anti-inflammatory effects of steroids are conferred by the GC
85 receptor (GR), a ligand-dependent transcriptional regulator that suppresses the expression of
86 inflammatory genes (3). Steroid receptor coactivator (SRC)/p160 family members (SRC1,
87 SRC2/Transcriptional Intermediary Factor 2 (TIF2)/GR Interacting Protein-1 (GRIP-1), and
88 SRC3/Receptor-Associated Co-activator 3 (RAC3)/p300/CREB binding protein (CBP)-co-
89 integrator protein (pCIP)/Amplified in Breast 1 (AIB1)) serve as co-activators for all nuclear
90 receptors including GR. As such, this family of proteins interacts with ligand-bound GR to
91 recruit histone acetyltransferases (CBP/p300) and coactivator-associated arginine
92 methyltransferase 1 (CARM1) that unpack the condensed chromatin, thereby facilitating the

access of transcription factors to target genes. Unlike other p160s, GRIP-1 also possesses a unique GR co-repressor activity facilitating GC-mediated repression of Activator Protein (AP)-1 and Nuclear factor-kappaB (NF-κB) activities (19, 20). Although, originally identified as a nuclear receptor co-factor, GRIP-1 was later shown to engage in physical and functional interactions with an IRF family member, IRF-3, and serves as an IRF-3 co-activator in macrophages (21). Yet, the role of GRIP-1 in ASM cells has not been assessed. Here we characterized whether GRIP-1 is a component of the IRF-1 transcriptional regulatory complexes and whether such interactions affect the response of ASM cells to GCs.

MATERIALS AND METHODS

ASM Cell Culture and Characterization. Primary human ASM cells were isolated from the trachealis muscle of lung transplant donors and purified as described (22) in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings.

Transfection of ASM Cells. ASM cells were transfected using Basic Nucleofector Kit for Primary Smooth Muscle Cells according to manufacturer's instructions using Amaxa Nucleofector II device (program U-25) (Amaxa Biosystems, Cologne, Germany) (14). Small interfering RNA experiments were performed using siRNA Test Kit for Cell Lines and Adherent Primary Cells according to manufacturer's instructions (Amaxa Biosystems) (14). ASM cells were transfected (14) using 2 μg of different constructs (IRF-1 was kindly provided by Dr. Yokosawa, Faculty of Pharmaceutical Sciences, Hokkaido University, Japan (23); GRIP-1 was constructed as described previously (21)), and/or 2 μg of GC responsive element (GRE)-dependent luciferase reporter plasmid (Clontech Laboratories Inc., Mountain View, CA), and/or

2 μg of IRF-1–dependent luciferase reporter plasmid (Panomics, Inc., Fremont, CA), and/or 100 nM with the combination of three different Silencer Pre-designed small interfering RNA (siRNA) GRIP-1 (CACAGGAAUGAUUGGUAA, GGAGAUUGAUAGAGCCUUA, GACAAGGGUUGAAUAUGA) (Santa Cruz Biotechnology, Santa Cruz, CA), and 1 μg of β-galactosidase vector (used to normalize transfection efficiency) (Promega, Madison, WI). Controls included the parallel use of pcDNA3 empty vector (Stratagene, La Jolla, CA) and/or scramble nonsilencing siRNA (Santa Cruz Biotechnology). The activities of luciferase and β-galactosidase were evaluated using luciferase and β-galactosidase detection kits (Promega), respectively, according to the manufacturer's instructions (14). The reporter luciferase activities were normalized to β-galactosidase activity and expressed as relative luminescence unit (RLU). Data were then expressed as percentage of controls (means ± SEM).

Immunoblot Analysis. Immunoblot analysis for GRIP-1, IRF-1 and GR was performed as described previously (14). To ensure equal loading, the membranes were stripped and reprobed with anti–β-actin antibody (Santa Cruz Biotechnology). Immunoprecipitations using the IRF-1, GRIP-1 (Santa Cruz Biotechnology) and GR antibodies (Affinity BioReagents, Golden, CO) were performed as previously described (14).

***In vitro* Binding**. Glutathione S-Transferase (GST)-tagged IRF-1 was expressed in *Escherichia coli*, purified and tested for its ability to interact with *in vitro* transcribed/translated GRIP-1 derivatives as previously described (21). The gels were stained with Coomassie blue (not shown) and autoradiographed.

RT-PCR Analysis. Total RNAs were extracted from human ASM cells using RNeasy Mini Kit (Qiagen, Valencia, CA) as previously described (14) using CD38, MPK-1 and GAPDH primers as reported earlier (14, 24, 25). GRIP-1 primers were purchased from Santa Cruz Biotechnology.

In preliminary experiments, we determined, for each primer pair, the melting temperature and number of amplification cycles necessary to yield the appropriate PCR product size. Subsequently, only one representative gel within the linear range is presented. The semi-quantitative PCR approach was performed in parallel analyzing the intensity of the area density of each PCR band using a Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD), and the PCR results are expressed as a ratio of area density of MKP-1 or CD38 to GAPDH. All experiments were performed in three different cell lines.

Materials and Reagents. Tissue culture reagents were obtained from Invitrogen (Carlsbad, CA). Human recombinant (r) TNFα and rIFNγ were provided by Roche Diagnostics (Indianapolis, IN). Fluticasone propionate (FP) was purchased from Sigma (St. Louis, MO).

Statistical Analysis. Data points from individual assays represent the mean values of triplicate measurements. Significant differences among groups were assessed with analysis of variance (Bonferroni-Dunn test) or by t test analysis, with values of $P < 0.05$ sufficient to reject the null hypothesis for all analyses. Each set of experiments was performed with a minimum of three different human ASM cell lines.

RESULTS AND DISCUSSION

GRIP-1 is essential for GR-dependent gene transcription in ASM cells. Although GCs suppress activities of some but not all cytokine-induced inflammatory genes (11), the underlying molecular mechanisms appear complex since GC actions are highly promoter- and cell type-specific (13). Although GRIP-1 is a well-established GR receptor co-activator, its role in GC function is also complex as it can affect both GR transactivation and transrepression activities (19). As shown in Figure 1A, GRIP-1 was expressed in ASM whole cell extracts, and that

expression was unaffected by cytokine and/or steroid treatments. Our finding is in agreement with previous studies showing the conserved expression of GRIP-1 in a variety of smooth muscle cells including smooth muscle of gastrointestinal and urinary tracts, uterus, epididymis, prostate and bronchioles of murine tissues (26). Interestingly, we observed a physical interaction between endogenous GR and GRIP-1, and FP treatment significantly enhances this association (Figure 1B) suggesting that GRIP-1 recruitment to GR is a prerequisite step to initiate GR transcriptional activity. Whether GR also binds to other p160 members of the SRC family, as suggested by others (27), remains to be further investigated in ASM. Moreover, we provide the first demonstration of a role of GRIP-1 in GR-dependent gene expression in ASM cells. Indeed, the activity of a reporter construct containing luciferase reporter gene driven by GRE motifs was induced by FP, and GRIP-1 siRNA (but not control siRNA) partially decreased such induction (Figure 1C, top). Of note, specific siRNA GRIP-1 dramatically and efficiently reduced GRIP-1 protein contents (by 91%, Figure 1C, bottom). siRNA-GRIP-1, but not control siRNA, also reduced by more than 50% FP-induced expression of Mitogen-activated Protein Kinase Phosphatase 1 (MKP-1) (24, 28, 29) (Figure 1D). This observation provides the first physiological relevance of GRIP-1 in driving the expression of GC-inducible genes in airway structural cells. This finding is also clinically relevant as MKP-1 has recently gained a lot of attention as a factor mediating GC inhibition of a number of pro-asthmatic responses in ASM including expression of inflammatory genes IL-6, GRO-α, and CD38 (24, 28, 29) as well as cell proliferation (30). By regulating the transactivation of GR-inducible proteins (31), our study identifies GRIP-1 as a critical factor in driving the anti-inflammatory actions of GC in ASM.

IRF-1-induced transcription requires GRIP-1. We recently developed a cellular model of steroid insensitivity where GC-induced suppression of inflammatory genes (CD38,

fractalkine) was drastically reduced in the presence of pro-asthmatic cytokines TNFα/IFNγ (8, 14, 25, 32). Cytokine-induced GC insensitivity was mediated, at least in part, by the transcription factor IRF-1 (14) that interfered with GR transactivation activities, although the underlying mechanisms remain unknown. We first examined whether IRF-1 interacts with GRIP-1. Full-length IRF-1 fused to GST was tested for its ability to bind different GRIP-1 derivatives including: Nuclear Receptor Interaction Domain (NID) (aa 565-765), known to bind GR (19, 20), 2-repression domain (RD) (aa 648-1007) containing nuclear receptor (NR) boxes 2 and 3 and the co-repression domain, 3-RD (aa 715-1007) containing NR box 3 and the co-repression domain and RD alone (aa 765-1007) (Figure 2A, top). Interestingly, all but the NID interacted with IRF-1 (Figure 2A, bottom) suggesting a direct GRIP-1:IRF-1 interaction and defining RD as the minimal fragment of GRIP-1 that associates with IRF-1. In addition, the interaction between GRIP-1 and IRF-1 was further confirmed by co-immunoprecipitation using ASM whole cell lysate (Figure 2B). Such interaction was weakly detected in basal but dramatically enhanced after cytokine treatment. Interestingly, the fact that GRIP-1:IRF-1 interacts *in vitro* without any inducer, raising the interesting hypothesis that the close proximity of IRF-1 with GRIP-1 is the main factor that will drive such interaction. Unless cells are exposed to cytokines that leads to IRF accumulation in the nucleus, not much interaction with GRIP1 can be expected under basal conditions where IRF-1 expression is minimal and mostly in the cytoplasm. This is certainly true for IRF3 (Reilly et al., EMBO J. 2006 Jan 11;25(1):108-17) and IRF-1 ((Tliba et al., J Biol Chem. 2003 Dec 12;278(50):50615-23)), IRF-7, and IRF-9 (Rogatsky, unpublished data). It seems, however, unlikely that GRIP-1:IRF-1 *in vivo* interaction requires GR. Indeed, our initial co-imunoprecipitation studies failed to detect any interaction between IRF-1 and GR in ASM cells treated with cytokines and/or glucocorticoids (Tliba, unpublished data). Similarly, in

macrophages, Reilly et al., (EMBO J. 2006 Jan 11;25(1):108-17) and Ogawa et al., (Cell. 2005 Sep 9;122(5):707-21) were also unable to detect any interaction between IRF-3 and GR.

We next investigated whether GRIP-1 recruitment to IRF-1 is functional and investigated whether manipulating GRIP-1 levels modulates IRF-1 transactivation activities. The use of silencing strategies demonstrated that the siRNA to GRIP-1 (but not siRNA controls) reduced by more than 57% TNFα/IFNγ-induced IRF-1-dependent reporter gene activity (Figure 2C). GRIP-1 depletion also abrogated the expression of CD38, an IRF-1-dependent gene in ASM cells (14) as shown by the 47% reduction of cytokine-induced CD38 mRNA up-regulation (Figure 2D). Although SRC family members interact with other transcription factors and regulators such as AP-1, Serum Response Factor (SRF), NF-κB, CREB, and as muscle-specific factor, myocyte enhancer factor 2C (MEF2C) (20, 31, 33-35), our findings are the first to demonstrate that GRIP-1 also interacts with IRF-1 and modulates IRF-1 transactivation activities. Together, these observations suggest that IRF-1-induced transcription requires GRIP-1 and raise the hypothesis that an excess of IRF transcription factors could potentially affect GC function by competing with GR for GRIP-1 required for GC transcriptional functions.

Recruitment of GRIP-1 by IRF-1 mediates cytokine-induced GR dysfunction. We previously showed that inflammatory cytokines (TNFα and IFNγ) promote steroid insensitivity in ASM cells by inhibiting GR transactivation activity, an effect mediated by IRF-1 (14). Interestingly, GR competition for GRIP-1 was shown to be critical in antagonizing IRF-3-regulated transcription in macrophages (21). We therefore examined whether the presence of an excess of GRIP-1 would restore cell sensitivity to steroids diminished by cytokine treatment. As reported previously (14), treatment of ASM cells with TNFα/IFNγ combination strongly suppressed FP-dependent gene expression as shown by the reduced FP-induced GRE-luciferase

reporter activity in vector-transfected cells (Figure 3A). This suppression, however, was no longer observed in cells ove-rexpressing GRIP-1 (Figure 3A). Similarly, when steroid insensitivity was induced by over-expressing IRF-1, increased levels of GRIP-1 restored GR reporter activity in IRF-1-transfected cells (Figure 3B). Of note, GRIP-1 over-expression dramatically and efficiently increased GRIP-1 protein contents (by 2.2 fold, data not shown). These findings suggest that inflammatory cytokines promote GC insensitivity via activation of IRF-1 that associates GRIP-1 and sequesters GRIP-1 away from GR. This represents a potential molecular mechanism underlying steroid insensitivity induced by inflammatory cytokines. Whether GRIP-1 acts directly or indirectly through the recruitment of additional co-regulators to overcome IRF-1 regulation of GR signaling needs further investigation. Further, since GRIP-1 depletion inhibits only partially FP-transactivation activities (Figure 1C, 1D), additional studies are also needed to examine the possible redundant and/or overlapping functions of other SRC family members and specifically define whether other p160 co-factors drive GR transcription and whether such pathways contribute to the anti-inflammatory actions of GCs in ASM cells. Importantly, while the expression of IRF-1 has been demonstrated in the airway epithelium of asthmatics (Sampath D et al J Clin Invest. 1999 May;103(9):1353-61), no study has yet investigated whether the expression of IRF-1 or GRIP-1 is present in CS resistant asthmatics this will be the focus of our future investigations.

Conclusion: This study reveals a potential mechanism for the mutual antagonism observed between IRF-1 and GR in their ability to activate gene transcription. As a shared co-factor required for maximal gene activation by both transcription factors, limiting the amount of GRIP-1 available to GR in the presence of high level of IRF-1 would dramatically affect GC function. Our study also shows that the induction of anti-inflammatory proteins such as MKP-1

by GCs represents a GRIP-1-dependent response. In inflammatory diseases such as asthma where structural cells such as ASM are exposed to multiple pro-inflammatory factors, an abnormal increase in IRF-1 levels would not only lead to increased IRF-1-dependent inflammatory proteins but would also impair tissue steroid responsiveness by decreasing GC function.

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FIGURE LEGENDS

Figure 1: ASM expresses a functional GRIP-1. *(A & B)* Cells were treated for 6 hours with FP (100 nM) and/or TNFα (10 ng/ml) and IFNγ (500 UI/ml), then lysed and assayed for GRIP-1 by immunoblot analysis (*A*) or immunoprecipitated with anti-GR antibody, and probed for GRIP-1 by immunoblot analysis (*B*). *(C)* ASM cells co-transfected with GRE-luciferase reporter and

either GRIP-1 siRNA or control siRNA were exposed to 100 nM of FP for 6 hours. Luciferase activities were expressed as percentage of control and assessed as described in the Methods section (top). In parallel experiments, total cell lysates were assayed for GRIP-1 by immunoblotting (bottom, upper gel). The nitrocellulose membrane was then stripped and blotted again for β-actin for equal loading control purpose (bottom, lower gel). *, $P < 0.05$ compared with untreated cells transfected with control siRNA; $^{\#}$, $P < 0.05$ when compared with FP-treated cells transfected with control siRNA. (*D)* Total mRNA was isolated from cells treated with FP for 6 hours as shown, and subjected to RT-PCR with MKP-1, GRIP-1 and GAPDH primers (top). Bottom, scanning densitometry of three representative RT-PCR gels of MPK-1 with each condition normalized over the area density of the corresponding GAPDH content. The results were expressed as fold increase over untreated conditions. *, $P < 0.05$ compared with untreated cells transfected with control siRNA; $^{\#}$, $P < 0.05$ when compared with FP-treated cells transfected with control siRNA.

Figure 2: GRIP-1 is novel co-activator in IRF-1 regulation of gene transcription in ASM cells. *(A) Top panel*, a diagram of GRIP-1 derivatives tested for binding to GST-IRF-1 including NID, 2-RD, 3-RD and RD alone, as indicated. *Bottom panel*, mapping of the interacting surfaces on GRIP-1. *In vitro*-produced GRIP-1 derivatives from panel A were tested for their ability to interact with GST-IRF-1. *(B)* Cells stimulated with TNFα (10 ng/ml) and IFNγ (500 UI/ml) for 6 hours, as shown, were lysed, immunoprecipitated with anti-IRF-1 antibody, and assayed for GRIP-1 by immunoblot analysis**.** *(C)* ASM cells co-transfected with IRF-1-luciferase reporter construct and either 100 nM of GRIP-1 siRNA or control siRNA were exposed to TNFα (10 ng/ml) and IFNγ (500 UI/ml) for 6 hours. The results were expressed as fold increase over basal. The luciferase activity was assessed as described in Methods. *, $P < 0.05$ compared with

untreated cells transfected with siRNA control. *(D)* In parallel experiments, total mRNA was subjected to RT-PCR with CD38, GRIP-1 and GAPDH primers (top). Bottom, scanning densitometry of three representative RT-PCR gels of CD38 with each condition normalized over the area density of the corresponding GAPDH content. The results were expressed as the fold increase over untreated cell values. *, $P < 0.05$ compared with untreated cells transfected with control siRNA; $^{\#}$, $P < 0.05$ when compared with FP-treated cells transfected with control siRNA.. **Figure 3: GRIP-1 over-expression reduces GR transcriptional activation from cytokine-/IRF1-dependent inhibition.** *(A)* ASM cells co-transfected with GRE-luciferase reporter and either 2 μg of full length GRIP-1 or pcDNA3 were exposed to TNFα (10 ng/ml) and IFNγ (500 IU/ml) for 6 hours and/or FP (100 nM) added 2 hours before. The luciferase activity was determined as described in Methods. *(B)* ASM cells co-transfected with GRE-luciferase reporter construct and 2 μg of full length GRIP-1 and/or 2 μg of full length IRF-1 were exposed to FP (100 nM) for 6 hours and luciferase activity was determined as described in Methods. Results were presented as percentage of control and were representative of three separate transfection experiments.*, $P < 0.05$ compared with untreated cells transfected with pcDNA3; **, $P < 0.01$ compared with untreated cells transfected with pcDNA3; $^{\#}$, $P < 0.05$ when compared with FP-treated cells transfected with pcDNA3; NS, not significant.

Figure 4: A model illustrating the role of IRF-1 in modulating steroid responsiveness in ASM cells. ***A,*** in steroid sensitive conditions where GCs increase the expression of protein MKP-1 to exert its anti-inflammatory action, GRIP-1 recruitment to GR on GRE sites of MKP-1 promoter is required as a part of GR activation complex. ***B,*** in steroid resistant conditions the impairment of GCs action by cytokine treatment is due to the fact that GRIP-1 is depleted from GR complex due to its recruitment on IRF-1 binding sites that leads not only and reduced GC action but also

410 to increased expression of the pro-inflammatory protein such as CD38. We therefore propose a
411 model where cell sensitivity to GC is determined by the balance between IRF-1 and GR levels
412 which both compete for the cofactor GRIP-1 that is essential for their transcriptional activities.
413 GC, glucocorticoid; GRE: glucocorticoid responsive elements; GR: glucocorticoid receptor.
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