SR Protein p30 Directs Alternative Splicing of Glucocorticoid Receptor Pre-mRNA to Glucocorticoid Receptor Beta in Neutrophils

Qing Xu,* Donald Y. M. Leung,*† Kevin O. Kisich†

*Division of Pediatric Allergy/Immunology, National Jewish Medical and Research Center Department of Pediatrics University of Colorado Health Sciences Center Denver, CO 80206

†Division of Basic Sciences, National Jewish Medical and Research Center Department of Immunology University of Colorado Health Sciences Center Denver, CO 80262

†Address Correspondence:
Donald YM Leung, MD, PhD National Jewish Medical and Research Center 1400 Jackson Street, Room K926 Denver, CO 80206 Tel: 303-398-1379 Fax: 303-270-2182 Email: leungd@njc.org
SUMMARY

Glucocorticoid (GC) insensitivity is a major clinical challenge in the treatment of many inflammatory diseases. It has previously been shown that GC insensitivity, in several inflammatory cell types, is due to an overabundance of the beta isoform of the glucocorticoid receptor (GCRβ) relative to the ligand binding isoform, GCRα. GCRβ functions as a dominant inhibitor of GCRα action. A number of both GCR isoforms are created from the same pre-mRNA transcript via alternative splicing, the factor or factors which control alternative splicing of GCR pre-mRNA are of great importance. In the current study, we have identified the predominant alternative splicing factor present in human neutrophils, which is known to be exceptionally GC insensitive. The predominant alternative splicing factor in neutrophils is SRp30c, which is one of several highly conserved serine-arginine rich proteins which are involved in both constitutive and alternative splicing in eukaryotic cells. Inhibition of SRp30c expression with antisense oligonucleotide strongly inhibited expression of GCRβ, and stimulated expression of GCRα. Antisense molecules targeted to other SR proteins had no effect. Our data indicate that SRp30c is necessary for alternative splicing of the GCR pre-mRNA to create mRNA encoding GCRβ.
INTRODUCTION

Glucocorticoid (GC)-insensitivity is a major clinical challenge in the treatment of chronic inflammatory diseases. The pharmacologic actions of GCs are mediated through intracellular receptors, the glucocorticoid receptors (GCR). There are two isoforms of GCR in human cells, GCRα and GCRβ, which are generated from a single gene via alternative splicing of the primary RNA transcript. Several studies indicate that GC-insensitivity has been associated with increased expression of GCRβ (1-7). GCRβ is truncated at the COOH terminus, which corresponds to the ligand binding domain. Thus, GCRβ cannot bind GC. In addition, GCRβ does not transactivate GC-sensitive genes, and functions as a dominant inhibitor of GCRα (8). Previous studies suggest that GCRα:GCRβ heterodimer formation may account for the reduced effectiveness of GC action in cells overexpressing GCRβ (8). Recent experiments in our lab demonstrated that overexpression of human GCRβ by mouse hybridoma cells results in the development of GC insensitivity by these cells (9).

Regulation of GCRβ expression is not well understood. Different cell types from the same individual can have very different ratios of GCRα to GCRβ. For example, freshly isolated peripheral blood neutrophils are GC insensitive. Both the absolute level of GCRβ, and the ratio of GCRβ to GCRα protein are much higher in neutrophils than in peripheral blood mononuclear cells (PBMC) from the same individuals (10). The ratio of GCRβ to GCRα can be altered by cytokine stimulation. After stimulation of neutrophils with IL-8, GCRβ mRNA levels increased remarkably and GCRα mRNA decreased to undetectable levels. Similarly, exposure of PBMC to IL-2 and IL-4 resulted in increased GCRβ expression and development of steroid insensitivity (1, 11). Since both GCRα and GCRβ are generated by alternative splicing of the same primary transcript (12-14), understanding the regulation of this splicing event is of primary importance for delineating mechanisms of GC insensitivity. Therefore, the goal of this research has been to identify the factor or factors involved in determining whether exon 9α is joined to exon 8 to
generate mRNA encoding GCRα, or whether exon 8 is joined to exon 9β, resulting in mRNA encoding GCRβ.

Pre-mRNA splicing is an essential step in eukaryotic gene expression. It is a multistep process including the accurate recognition of splice sites, excision of intronic sequences, and ligation of the 5' and 3' ends of the resulting fragments (15-19). The splicing process occurs with the assembly of a multicomponent structure known as the spliceosome (20). The major components of the spliceosome are small nuclear ribonucleoprotein particles (snRNPs) U1, U2 and U4/U6, and several proteins including a family of serine-arginine rich proteins known as the SR proteins.

SR proteins have a dual role in pre-mRNA splicing in vivo and in vitro (21). They are involved in constitutive as well as alternative splicing. SR proteins have one or two N-terminal RNA recognition motifs (RRMs) and a C-terminus rich in arginine/serine dipeptide repeats (the RS domain) (22) which mediates protein interactions with other components of the splicing machinery. In the following experiments, we have identified the SR protein required for alternative splicing of the GCR primary RNA transcript into GCRβ mRNA.
MATERIALS AND METHODS

Cell Isolation Human neutrophils and PBMC were isolated from normal healthy individuals using a Percoll (Amersham Pharmacia Biotech, UK) (23) density gradient. Forty ml venous blood was collected in syringes with 4.4 ml of 3.8% sodium citrate (Fisher Scientific, Pittsburgh, PA). The whole blood was centrifuged at 1200 RPM for 20 min, then upper Platelet Rich Plasma (PRP) layer was removed and centrifuged at 3000 RPM for 15 min. The Platelet Poor Plasma (PPP) supernatant was aspirated from the platelet pellet. In the meantime, 5 ml of 6% (wt/vol) dextran (Amersham Pharmacia Biotech, UK) and saline were added to 50 ml to the cellular component. Erythrocytes were allowed to sediment for 30 min at room temperature and the leukocyte-rich supernatant was collected. Then the leukocyte-rich supernatant was sedimented at 1000 RPM for 10 min. The supernatant was discarded and the WBC pellet was resuspended with 2 ml PPP. This was underlayed with 42% (wt/vol) Percoll, followed by 51% (wt/vol) Percoll. After 5 min the leukocytes were centrifuged at 1000 RPM for 10 min. PBMC and neutrophils formed separate bands at the Percoll density interfaces, and were collected. The purity of neutrophils isolated by this method was > 95%, as determined by Wright-Giemsa staining.

Stimulation of neutrophils with IL-8 Freshly separated neutrophils from normal individuals were cultured at a concentration of 1.0 x 10^6/mL in the presence and absence of IL-8 (0.5 µg/mL) (R&D systems, Minneapolis, MN) for two hours. Then cells were harvested and proteins were extracted.

Generation of mAbs: Hybridomas CRL-2385 (ATCC Rockville, MD) which produce monoclonal antibody against a conserved epitope on a subset of SR proteins were cultured for 10 days and then supernatants were collected (24).
**Western-blot Analysis** Cell and nuclear lysates were prepared from neutrophils, PBMC and the PLB-985 cell line. Cells were resuspended in buffer containing 10 mM Tris-HCl (pH 8.3), 1 mM EDTA and a mixture of protease inhibitors (0.1 mM PMSF, 1 µg/ml aprotinin, 1 µM pepstatin and 1 µM leupeptin). After 30 min, cells were centrifuged at 12,000 rpm for 10 min at 4°C. Supernatants were collected and proteins were resolved by electrophoresis, followed by electrophoretic transfer to PVDF membranes (BioRad, Richmond, CA) in Tris-glycine buffer. Membranes were blocked overnight with blocking buffer (5% milk, 10% 50 mM sodium phosphate, 3% 5M NaCl and 0.05% Tween 20) then incubated with primary antibody to GCRβ (rabbit polyclonal antibody, ABR Affinity Bioreagents INC, Golden, CO), total GCR (Santa Cruz Biotechnology, Santa Cruz, CA) and SR proteins (ATCC, CRL-2385) for 2 hours, washed and then incubated with secondary antibody - HRP Protein A (Amersham Pharmacia Biotech, UK) for GCRβ and total GCR, HRP-Rat Anti Mouse IgG1 (Zymed, So. San Francisco, CA) for SR proteins for 1 hour. Membranes were washed and developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech, UK).

**PLB-985 cell differentiation** PLB-985 cells were stimulated with retinoic acid (Sigma Aldrich, St. Louis, MO) (25) 14.9 µl 6.7 mM retinoic acid were added to 100 ml media with 2.0×10^7 cells (final concentration of retinoic acid 1 µM). Media were changed on the third day. After five days, slides were made and stained with Diff Quick (Fisher Scientific) to evaluate nuclear morphology.

**Transfection of antisense oligonucleotides using electroporation.** PLB-985 cells were washed once in HBSS. 5.0×10^6 cells were resuspended in 500 µl RPMI with 10% (vol/vol) FCS (without antibiotics), and then transferred to electroporation cuvettes (Disposable Micro-Electroporation Chambers, Life Technologies, Gaithersburg, MD). 10 µg of fluorescein-labeled antisense oligonucleotides (Midland Certified Reagent Co, Midland, TX) were added to 500 µl RPMI/FCS in the cuvettes, mixed gently, and then incubated at room temperature for 10 min.
Cells were permeabilized with a single pulse using the GIBCO BRL electroporation apparatus (Life Technologies, Grand Island, NY) set to 800 Ω 230V. These conditions were defined as producing optimal permeabilization with minimal toxicity in preliminary experiments. The cells were incubated for 25-30 min at room temperature in the electroporation cuvettes and then transferred to flasks containing 25 ml differentiation media.

**Flow cytometry** Flow cytometry was performed on a FACSCaliber instrument, and analysis used CELL QUEST Software (Becton Dickinson). Uptake studies were performed using purified oligonucleotides to ensure that only intact oligonucleotides were studied. 1 ml containing 1.0×10⁶ cells was added into 5 ml FALCON tubes (Becton Dickinson Labware, Franklin Lakes, NJ), and efficiency of uptake was checked by the Flow Cytometry. Cells positive for fluorescein were sorted within 12 hours of electroporation, and placed into cultures for differentiation.

**RNA isolation and cDNA preparation** Total RNA was isolated from fresh neutrophils, PBMC and PLB-985 cells by using RNA-Bee (TEL-TEST, Friendswood, TX) according to the manufacturer’s instructions. Reverse transcription reactions used ≈2 µg of total RNA in 20 µl with 200 units of SuperScript II Reverse transcriptase (Invitrogen, Carlsbad, CA).

**Plasmid construction** Plasmids used to create standard curves for realtime PCR were generated as follows: SRp30a cDNA region spanning nucleotides 111-990 (NM-006924) was amplified with forward primer (5' − TTTTCGTCACCGCCATGTC−3') and reverse primer (5' − CAATTCAACACTTTAGCCA−3')(all primers were designed using Macvector 6.5.3)(Accelrys), SRp30b region spanning nucleotides 194-630 (NM_003016) was amplified with forward primer (5' − GACCTCCCTCAAGGTGGACAAC−3') and reverse primer (5' − ACCGAGATCGAGAACGAGTGC−3'), SRp30c cDNA region spanning nucleotides 312-986 (NM_003769) was amplified with forward primer (5' − CCAGGACTTATGGAGGTCGG−3') and reverse primer (5' − AACCCCAAAAGACAGACG−3') using cDNA prepared from RNA
extracted from fresh cells. The PCR products were cloned into the PGEM-T vector (Promega, Madison, WI).

**Real-time PCR** An ABI7700 (Perkin Elmer Biosystems, Foster City, CA) with Taqman probe sets was used to quantify mRNA levels. Primers were designed to amplify segments of around 200bp to maximize efficiency. Primer sequences were as follows: SRp30a: forward primer: 5' - TTGAGTTGCAGGACCAGC - 3'. **Reverse primer:** 5' - CGTAATCATAGCCGTCGCG - 3'. Taqman probe: 6-fam-CGCGGAAGACGCGGTGTATG-tam. SRp30b: forward primer: 5' - CGCGGCTTCGCCTTC - 3'. **Reverse primer:** 5' - ATGGCATCCATAGCGTCCTC - 3'. Taqman probe: 6-fam-TTCGCTTTTCACGACAAGCGCGAC-tam. SRp30c: forward primer: 5' - TTTCCAGTTCTTTGTTTGAGGAC - 3'. **Reverse primer:** 5' - CTGCATGTGATCCTTCAGGT - 3'. Taqman probe: 6-fam-CCTCCGTCAGGCAGCTGCGCAG-tam. Each sample was tested in duplicate and all PCR runs were performed three times. Each sequence was quantified relative to a standard curve of its cognate cloned cDNA sequence.
RESULTS

SR protein levels in neutrophils and PBMC

In order to identify candidate proteins for the GCR alternative splicing factors, we compared the complements of SR proteins in cell extracts from human neutrophils, which are rich in GCRβ, with extracts from PBMC, which have low levels of GCRβ. Western-blot analysis of cell extracts was performed on 20 µg of nuclear extracts from neutrophils and PBMC separated from six normal donors. Both freshly isolated neutrophils and PBMC expressed SR proteins (Figure 1). The blots were developed using chemiluminescence, and the intensity of each band was measured using a flatbed scanner and NIH Image 1.61. We compared SRp20, SRp30, SRp40, SRp55, and SRp75 levels in neutrophils and PBMC. However, neutrophils contained SR proteins predominantly in the 30 kDa range. Levels of SRp30 in neutrophils were significantly greater than in PBMC when data from the six individuals were pooled and averaged [3.3±0.97 density units (DU) vs 1.3±0.42 DU (mean±SEM); p=0.02; t test]. Ratios of SRp30 in neutrophils vs PBMC for the six donors ranged from approximately 1 (donor three) to 6.7 (donor four). In order to identify the specific SRp30 species present in human neutrophils, we utilized realtime PCR and specific Taqman probe sets for SRp30a, SRp30b, and SRp30c. Figure 2 shows that only mRNAs encoding SRp30a and SRp30c were present in the neutrophils. Levels of SRp30c mRNA were approximately 23-fold higher (p=0.03, t-test for paired samples) than levels of SRp30a. This suggests that SRp30c is probably the predominant SR protein present in human neutrophils.

SR protein levels in neutrophils stimulated with IL-8

We have previously demonstrated that IL-8 enhances GC insensitivity in neutrophils by increasing synthesis of GCRβ relative to GCRα (10). Therefore, we performed Western analysis on extracts from neutrophils which had been treated with IL-8 compared to unstimulated neutrophils (Figure 3A). Freshly isolated neutrophils were treated with IL-8 or medium only for
two hours. 20 µg of nuclear extracts from neutrophils separated from four normal donors were separated on SDS-PAGE gels. After transferring to PVDF membranes and staining with specific anti-SR monoclonal antibody, the blots were developed with HRP conjugated secondary antibody and chemiluminescence. Densitometry of the films revealed that SRp30 levels increased significantly in neutrophils treated with IL-8 compared to neutrophils incubated in medium only (62±14 DU vs 32±10 DU; \( p=0.04 \)) (Figure 3B). Therefore, conditions which result in increased GCRβ levels in neutrophils also result in increased levels of SRp30 proteins in neutrophils.

**PLB-985 cell differentiation**

In order to obtain functional evidence of the identity of the GCR alternative splicing factor, we initially attempted to specifically inhibit SRp30a and SRp30c expressions in neutrophils using antisense phosphorothioate oligonucleotides. However, due to the brief viability of these cells in culture we were unable to obtain reproducible results. Therefore, we employed a model of neutrophil-like differentiation using the PLB-985 cell line (25). Cells of this line are capable of granulocytic maturation to become neutrophil-like in the presence of inducing agents such as retinoic acid. After exposure to retinoic acid, the cells become neutrophil-like. Figure 4 illustrates the morphology of PLB-985 cells after five days of exposure to retinoic acid.

**GCRα and GCRβ levels in PLB-985 cells before and after neutrophilic differentiation.**

In order to measure the dynamics of GCR expression upon differentiation of PLB 985 cells, we performed Western-blot analysis of GCR from lysates of cells prior to and after five days of retinoic acid exposure. Figure 5 revealed that GCRα and GCRβ levels both increased upon exposure to retinoic acid relative to undifferentiated PLB-985 cells. However, densitometry revealed that GCRβ levels (5.2±2.4 DU on day 0 vs. 143.2±18.2 DU on day five, \( p=0.01 \)) increased much more than GCRα (1.82±0.55 DU on day 0 vs. 18.03±4.05 DU on day
five, \( p = 0.04 \). This suggests that both synthesis of GCR primary transcript, and alternative splicing are increased upon exposure to retinoic acid, but direction of alternative splicing towards GCRβ.

**SR protein levels in undifferentiated and differentiated PLB-985 cells**

In order to examine our hypothesis that enhanced alternative splicing of GCR pre-mRNA is associated with enhanced expression of SR proteins in the 30 kDa range, we performed Western analysis on cell extracts prior to and after five days of exposure to retinoic acid. Densitometry of the film shown in Figure 6A shows that SRp30 levels significantly increased after differentiation relative to undifferentiated cells. Figure 6B shows the density units of SRp30 from three experiments before and after differentiation (1.08±0.25 DU vs. 77.47±19.30 DU, \( p = 0.02 \)). Realtime PCR revealed that upon differentiation PLB-985 cells expressed increased levels of SRp30a, SRp30b, and SRp30c. The expression amounts for SRp30a and SRp30c were much higher than those found in primary neutrophils at \( 4.3 \times 10^5 \) pMol/ng 18s and \( 3.24 \times 10^4 \) pMol/ng 18s, respectively, while mRNA for SRp30b was present at approximately \( 1.2 \times 10^5 \) pMol/ng 18s (data not shown). Expression of SRp30b was in contrast to data obtained from primary neutrophils, which did not express SRp30b.

**Inhibition of SRp30c via antisense phosphorothioate oligonucleotides inhibits alternative splicing of GCR pre-mRNA.**

In order to determine which of the three SRp30 proteins present in PLB-985 cells were necessary for alternative splicing of the GCR pre-mRNA to generate GCRβ, each SRp30 protein was specifically targeted with antisense phosphorothioate oligonucleotides. Electroporation of fluorescein-tagged antisense oligonucleotides for SRp30a, SRp30b or SRp30c were performed as described, and the cells were sorted for the fluorescein positive population. The transfected cells were then returned to cultures and exposed to retinoic acid for up to five days. Nuclear extracts were then prepared and separated by SDS-PAGE for Western analysis of GCRβ protein levels.
(Figure 7). Figure 7A is a representative Western-blot showing that GCRβ levels were inhibited for up to 48 hours with antisense oligonucleotide for SRp30c after transfection relative to control oligonucleotide. Figure 7B shows the density units of GCRβ from three experiments transfected with antisense oligonucleotide for SRp30a, b, c, and control oligonucleotide. GCRβ levels were significantly inhibited at 24 hours after transfection with antisense oligonucleotide for SRp30c compared to control oligonucleotide (19.43±11.98 DU vs. 153.1±15.4 DU, p=0.003). As shown in figure 8, GCRα was simultaneously elevated following treatment of the cells with anti-SRp30c relative to cells treated with control oligonucleotide. In the cells transfected with antisense oligonucleotides for SRp30a, or b there was no effect on GCRβ levels relative to control oligonucleotide, or untreated cells.

DISCUSSION

Pre-mRNA splicing is an essential step in gene expression by eukaryotes. It is a multistep process including the accurate recognition of splice sites, excision of intronic sequences, and ligation of the resulting fragments back into a single molecule. All of these steps are performed by a multi-molecular complex of proteins and RNA known as the spliceosome (20). Recognition of the 5' and 3' splice sites by the spliceosome requires several small nuclear ribonucleoprotein particles (snRNPs), including U1, U2 and U4/U6 and the non-snRNP proteins including the family of serine/arginine-rich (SR) proteins (27-30). Around 40% of the human genes may be constitutively spliced (31). The selection of splice sites is determined by several parameters including the proximity and strength of splicing signals (32). Constitutive splicing can be performed by the minimal spliceosome, and involves ligation of the distal end of the upstream exon with the nearest splice acceptor, which is at the proximal end of the next exon in the sequence. Among the different mechanisms of constitutive and alternative splicing, the possible exclusion of sequences where both the 5' and the 3' intronic junctions are located within an exon represents a further pattern of alternative splicing (33). In alternative splicing, one or
more splice acceptors and associated exons are skipped, resulting in removal of their coding information from the final mRNA. In the case of GCR, exon 9α is skipped and exon 9β is ligated directly to the distal end of exon 8. This results in inclusion of an alternative COOH terminus to the GCR. The COOH terminus encoded by exon 9α included the ligand binding domain, while that encoded by exon 9β includes a domain which cannot bind GC. Stimulation of alternative splicing for other mRNAs such as fibronectin (34–36), beta-tropomyosin (37), Apolipoprotein B (apoB) (38), adenylyl cyclase stimulatory G-protein G alpha(s) (39) and survival motor neuron (SMN) (40) requires the coordinated action of specific SR proteins and several classes of cis acting mRNA elements including purine-rich splicing enhancers known as exonic splicing elements (ESE). These proteins bind and can direct the splicing to alternative splice sites in a concentration-dependent manner, regulate the stability of mRNA and have a general role in mRNA export (41–43). SR proteins contain one or two N-terminal RNA recognition motifs (RRMs), which mediate binding to the ESE in the RNA, and a C-terminus rich in arginine-serine dipeptide repeats (the RS domain) which mediate protein interactions with other components of the spliceosome. Alternative splicing is a common mechanism to create alternative protein isoforms. A characteristic of alternative splicing is to introduce stop codons or frame shifts that can either switch off genes or create proteins with different C termini.

The strategy used in these studies to identify the factor or factors involved in selection of the alternative splice site in exon 9β was to first compare the complement of SR proteins present in cells rich in GCRα, such as PBMC, compared with SR proteins present in cells rich in GCRβ, such as neutrophils. This comparison, shown in figure 1, revealed that while PBMC contain a variety of SR proteins, neutrophils contain predominantly SR proteins in the 30 kDa range, with minor bands representing SRp75 and SRp20. The strong band near 30 kDa could contain one or more of SRp30a, SRp30b, or SRp30c. Of these three candidates, SRp30c has been found to stimulate alternative splice site selection in CD45 pre-mRNA in leukocytes (44), while SRp30a (SF2/ASF) has been shown to influence alternative splice site selection in fibronectin (45, 46), beta-tropomyosin (37), influenza virus (47), and herpes-simplex virus, and adenovirus E1A pre-
mRNAs (48-51). SRp30b (SC35) has been found to influence splicing of CD45, and implantation related genes among others.

In order to identify which of the SRp30 molecules were present in neutrophils and could participate in alternative splicing of GCR pre-mRNA, we performed both RT-PCR and realtime PCR for the mRNAs encoding SRp30a, SRp30b, and SRp30c. Since SRp30b (SC35) was undetectable by either RT-PCR or realtime PCR, we concluded that the SRp30a and SRp30c were the most likely candidates for GCR alternative splicing factors. However, as SRp30c mRNA was present in neutrophils at much higher levels than SRp30a, we concluded that the majority of the protein present in neutrophils upon Western analysis was probably SRp30c.

Conclusive evidence for the identity of the GCR splicing factor or factors required the ability to specifically inhibit expression of SRp30a and SRp30c in a cell type which also expressed GCRβ. We initially attempted to use phosphorothioate antisense DNA molecules targeted to SRp30a and SRp30c to inhibit expression of these proteins in neutrophils, but the short lifespan of these cells in culture made it very difficult to obtain reproducible data. Therefore, we developed a model based of PLB-985 cells, which are from a promyelocytic leukemia that differentiate into neutrophil-like cells in response to retinoic acid (25).

Upon exposure to retinoic acid, PLB-985 cells begin to differentiate, which takes about five days to complete as shown in Figure 4. In addition, these cells express exclusively higher levels of SR proteins in the 30 kDa range after differentiation, which are undetectable prior to differentiation. Both GCRα and GCRβ increased during differentiation, however GCRβ increased by approximately 27-fold while GCRα increased by approximately 10-fold. Therefore, alternative splicing of GCR pre-mRNA is stimulated during differentiation of PLB-985 cells, coincident with a dramatic increase in SRp30 proteins. According to realtime PCR, PLB-985 cells express SRp30a, SRp30b, and SRp30c. Therefore, mRNAs encoding all three proteins were targeted using specific phosphorothioate antisense DNA molecules.

Alternative splicing of GCR pre-mRNA to GCRβ was not affected when cells were treated with control phosphorothioate oligonucleotides, nor was it affected when the
oligonucleotides specifically targeted SRp30a or SRp30b. However, as shown in Figure 7, targeting of SRp30c with a specific phosphorothioate antisense molecule resulted in a dramatic reduction in GCRβ production for approximately 24 hours after transfection, while GCRα production was stimulated (Figure 8). Therefore, SRp30c was necessary for alternative splicing of GCR pre-mRNA to generate mRNA encoding GCRβ. Since SRp30c represents the major alternative splicing factor present in neutrophils, we conclude that SRp30c is required for alternative splicing to generate GCRβ mRNA. Since GCRβ is intimately involved in GC insensitivity, and GCRβ is dependent on the presence of SRp30c, this SR protein may make an attractive target for therapeutic intervention.
LITERATURE CITED:

FOOTNOTES

1Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>apo B</td>
<td>Apolipoprotein B</td>
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<tr>
<td>DU</td>
<td>density units</td>
</tr>
<tr>
<td>ESE</td>
<td>exonic splicing enhancer</td>
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<td>GC</td>
<td>glucocorticoid</td>
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<td>GCR</td>
<td>glucocorticoid receptor</td>
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<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
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<td>PPP</td>
<td>Platelet Poor Plasma</td>
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<td>PRP</td>
<td>Platelet Rich Plasma</td>
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<td>RRM</td>
<td>RNA recognition motifs</td>
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<td>SMN</td>
<td>survival motor neuron</td>
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<td>snRNP</td>
<td>small nuclear ribonucleoprotein particles</td>
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<td>SR</td>
<td>serine-arginine rich</td>
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# Table I: Demographics of Asthmatic Subjects and Asthma Severity Measures

Including the Log_{10} IC_{50}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>White (n=180)</th>
<th>Black (n=66)</th>
<th>P value*</th>
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<tr>
<td>Age (yrs)</td>
<td>21.9±0.9</td>
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<tr>
<td>% Male</td>
<td>42.4</td>
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<tr>
<td>% Atopic</td>
<td>83.4</td>
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<td>Asthma Duration (years)</td>
<td>14.2±0.7</td>
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<td>FEV₁ (%Predicted)</td>
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<td>75.7±2.9</td>
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<td><strong>Asthma Severity Score</strong></td>
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<td>Inhaled GC Dose (µg/day)</td>
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<td>T lymphocyte GC response [Log_{10} IC_{50} (nM)]</td>
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<td>Nanomolar value</td>
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* One-way Anova \( t \) test and chi-square used for analysis. Values expressed as mean and the standard error of the mean. Significant \( P \) value is <0.05.

**Severity score calculated based the National Asthma Education and Prevention Program guidelines for asthma severity.\(^7\)

1. <2 days use per week or <1night/month with symptoms or reported albuterol
2. >2 days per week with symptoms or of reported albuterol use, <1night/week;
3. Daily symptoms and 1-3 nights/week of nighttime symptoms
4. Daily symptoms and >5 nights/week of nighttime wakening or steroid dependent (defined as requiring \( \geq 880 \) µg/day of inhaled fluticasone propionate or \( \geq 1200 \)g of budesonide; or oral GC use of for greater than 6months out of the past year.)\(^9\)
Table II: Correlations Between log$_{10}$ IC$_{50}$ Values and Measures of Disease Severity

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<th>White Asthmatics</th>
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<td>Asthma Duration</td>
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<td>Inhaled GC Dose</td>
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<td>0.23</td>
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</tr>
</tbody>
</table>

Pearson's correlation used to generate r values. P values are significant at p<0.05.
Figure Legend:

**Figure 1.** Expression of SR proteins in neutrophils and PBMC from six normal individuals. SRp30 protein levels, analyzed by Western-blot, are significantly higher in neutrophils as compared to PBMC. Data are expressed as mean ±SEM analyzed by two-tailed paired t-test.

**Figure 2.** Real-time PCR shows that SRp30c mRNA is significantly higher in neutrophils from four normal individuals compared to SRp30a and SRp30b. Data expressed as mean±SEM analyzed by two-tailed paired t-test.

**Figure 3.** SRp30 levels after IL-8 stimulation in neutrophils. A: Representative Western-blot of increased expression of SRp30 proteins in neutrophils treated for two hours with IL-8 vs media only in a normal individual. B: Mean densitometry of SRp30 proteins in neutrophils from four normal individuals. After two hours of stimulation with IL-8, SRp30 levels increased in neutrophils as compared to neutrophils in media alone. Data are expressed as mean ±SEM analyzed by two-tailed paired t-test.

**Figure 4.** Neutrophilic differentiation of PLB-985 cells. A. PLB-985 cells demonstrating large nuclei and intensely staining cytoplasm before differentiation. B. After differentiation, cells exhibit neutrophil-like morphology, including multilobed nuclei, and lightly staining cytoplasm.

**Figure 5.** Western-blot of total GCR in PLB cells before and after differentiation. After differentiation, expression of GCRα and GCRβ increased. But GCRβ level increased significantly more compared to GCRα level. A representative blot from three experiments is shown in Panel A and the mean ±SEM of three experiments are shown in Panel B analyzed by two-tailed paired t-test.
**Figure 6.** Expression of SR proteins in PLB cells before and after differentiation. After differentiation, SRp30 proteins selectively increased in differentiated PLB cells. A representative blot from three experiments is shown in Panel A and the mean ±SEM of three experiments are shown in Panel B. Data were analyzed by two-tailed paired *t*-test.

**Figure 7.** Expression of GCRβ in differentiated PLB cells transfected with control oligonucleotide and antisense oligonucleotides for SRp30a, b, c. Proteins were extracted from day 1, day 2 and day 3 after differentiation and before differentiation. SRp30a, antisense oligonucleotide significantly inhibited the expression of GCRβ at 24 hours after transfection, but not SRp30a or SRp30b or control oligonucleotides. A representative blot from three experiments is shown in panel A and the mean ± SEM of three experiments are shown in panel B. *GCRβ levels are significantly inhibited in cells on day one, with antisense oligonucleotide for SRp30c compared with control oligonucleotide. \( P = 0.003 \). Analyzed by two-tailed unpaired *t*-test.

**Figure 8.** Expression of GCRα in differentiated PLB cells transfected with control oligonucleotide and antisense oligonucleotide for SRp30c. Proteins were extracted from day 0 (prior to differentiation), day 1, day 2 and day 3 after differentiation. SRp30c, antisense oligonucleotide significantly stimulated the expression of GCRα relative to control oligonucleotide. A representative blot from three experiments is shown in panel A and the mean ± SEM of three experiments are shown in panel B. *GCRα levels are significantly stimulated in cells following treatment with antisense oligonucleotide for SRp30c compared with control oligonucleotide. \( P < 0.01 \). Analyzed by two-tailed unpaired *t*-test.
Figure 1
Figure 2
Figure 3
Figure 4

Before differentiation

After differentiation
Figure 5
Figure 6
Figure 7
Figure 8

A  Days of retinoic acid exposure

<table>
<thead>
<tr>
<th>Days</th>
<th>Control</th>
<th>Oligonucleotide</th>
<th>SRp30c Antisense</th>
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<tr>
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</tbody>
</table>

B  Density units of GCR alpha

- **control**
- **SRp30c**
SR protein p30 directs alternative splicing of glucocorticoid receptor Pre-mRNA to glucocorticoid receptor beta in neutrophils
Qing Xu, Donald Y.M. Leung and Kevin O. Kisich

J. Biol. Chem. published online May 8, 2003

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