Interaction between the Androgen Receptor and RNase L Mediates a Cross-talk between the Interferon and Androgen Signaling Pathways *$^\circ$

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Signaling by androgens and interferons (IFN) plays an important role in prostate cancer initiation and progression. Using microarray analysis, we describe here a functional cross-talk between dihydrotestosterone and interferon signaling. Glutathione S-transferase pull-down and co-immunoprecipitation experiments reveal that the androgen receptor and the interferon-activated RNase L interact with each other in a ligand-dependent manner. Furthermore, overexpression of wild type RNase L confers IFN sensitivity to a dihydrotestosterone-inducible reporter gene, whereas the wild type receptor did not. Based on our data we hypothesize that in 22Rv1 cells, activated androgen receptor (AR) contributes to the insensitivity to IFN of the cell. Accordingly, we show that AR knockdown restores responsiveness to IFN. Our findings support a model in which both the activation of AR and the down-regulation of IFN signaling can synergize to promote cell survival and suppress apoptosis. This model provides the molecular basis to understand how mutated RNase L can lead to early onset PCa and illustrates how inflammatory cytokines and nuclear hormone signaling contribute to tumor development.

Tumors are increasingly regarded as neoplastic organs with a specific microenvironment that comprises cells of different origins. The contribution of tumor-associated fibroblasts and endothelial cells to tumor growth is now well established (1). The role of inflammatory cells has received renewed attention with two recent reports that describe the interplay between tumor cells and tumor associated immune cells (2, 3).

In contrast to the contribution of inflammatory cytokines and immunoglobulins to tumor growth, T-cell-produced interferons have been shown to antagonize DHT-mediated transactivation. Furthermore, we show that AR-insensitive cells can become sensitive to IFN upon down-regulation of AR expression by siRNA.

We propose a model in which either activation of AR signaling by mutation or growth factors or down-regulation of IFN signaling through mutation of RNase L contribute to cell survival and suppression of apoptosis. This model sheds light on the effect of RNase L mutation on PCa progression. It also illustrates how inflammatory cytokines can affect hormonal signaling and therefore contribute to tumor development.

MATERIALS AND METHODS
Cell Culture and Transfection—The breast cancer cell line, MDA-MB-453, and the prostate cancer cell lines 22Rv1 and MDA-PCa2b, were purchased from ATCC and maintained in RPMI 1640 (Invitrogen) supplemented with 10% fetal bovine serum, Hepes, sodium pyruvate.

Cells were transfected in 96-well plates with a total of 150 ng of DNA and 0.5 μl of FuGENE (Roche Diagnostics) per well. The reporter to expression vector ratio was typically kept at 2:1. Empty pCDNA3.1 vector was used to maintain the amount expression vector constant. Cells were treated with DHT or IFNs 24 h after transfection. Luciferase activity was read 18 h post-treatment using Steadylite (PerkinElmer Life Sciences) following the manufacturer’s recommendations.

Cloning of RNase L cDNA—cDNA was synthesized and subcloned from MDA-MB-453 and 22Rv1 RNA extracted using TRIzol according to the manufacturer’s protocol (Invitrogen). Twelve independent clones were sequenced on both strands. Genomic zygosity was confirmed on PCR clones obtained from each cell line.

RESULTS AND DISCUSSION
To address whether IFNγ and androgen signaling pathways can functionally interact in a genome wide context, we performed a microarray analysis of RNA derived from AR-expressing PCa 22Rv1 cells or breast cancer MD-MB-453 cells that were treated for 16 h with vehicle, 10−7 M DHT, 500 units/ml IFNγ, or a combination of both. Both 22Rv1 and MDA-MB-453 cells responded to DHT in a comparable extent, with, respectively, 185 and 124 genes regulated more than 1.5× (p < 0.01) (Fig. 1A). Treatment with IFNγ gave a solid transcriptional response in MDA-MB-453 cells with 309 genes significantly up-regulated versus vehicle treatment (p < 0.01). In contrast, 22Rv1 cells responded to the IFNγ very weakly, with only 11 genes displaying 1.5–4-fold increased expressions (Fig. 1A). A microarray study performed in the AR expressing PCa cell line, MDA-PCa2b, showed a similar response to DHT and absence of sensitivity to IFNγ (data not shown). Notably, genes that poorly responded to IFNγ in both PCa cell lines, like Stat1, IRF1, and GBP1, were among the most responsive to IFNγ treatment in MDA-MB-453 cells (Fig. 1A and data not shown) suggesting that 22Rv1 cells are deficient in their response to IFNγ. Further analysis of data from MDA-MB-453 cells revealed a subset of genes that displayed a greater than 40% reduction (p < 0.02) of activation in the presence of both DHT and INFγ compared with either treatment alone (Fig. 1B). This observation was confirmed using QRT-PCR (Tagman) analysis. By this method we found that IFNγ could suppress DHT-mediated induction of the adrenocortical receptor 2b (ADRA2b) and of RASGEF1a by 90 and 60%.
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**Figure 1.** Mutual antagonism between DHT and IFN. A, number of genes regulated by 100 nM DHT, 500 units/ml IFNγ, or both in MDA-MB-453 or 22Rv1 cells. B, list of genes differentially regulated by combined versus DHT or IFN treatment. Genes represented here were down-regulated by at least 40% in each replicate of the combined treatment versus single treatment (p < 0.02). Three genes are shown in italics at the bottom of the table that were not affected by the presence of DHT. C, QRT-PCR confirmation of microarray analysis for the adrenergic receptor 2a (AD2a) (top), RasGEF1 (middle) and Caspase 1 (bottom). Data are representative of at least two independent experiments performed in triplicate. Error bars represent standard deviation. D, relative luciferase activity of MDA-MB-453 (left) or 22Rv1 (right) cells transfected with MMTV-Luc reporter construct and treated with dimethyl sulfoxide (DMSO) or 100 nM DHT in the presence of increasing doses of IFNγ.

respectively (Fig. 1C). Conversely, DHT antagonism on IFNγ signaling was also observed. Under conditions where DHT alone did not affect gene expression, it could oppose IFNγ-mediated induction of a subset of genes by greater than 40% (p < 0.02). For example, our microarray data identified Caspase 1 as one of these genes. Confirmation using QRT-PCR showed that DHT almost abrogates the induction of Caspase 1 by IFNγ (Fig. 1C). Taqman analysis also suggested that both the degree of DHT and IFN induction and the level of antagonism appeared to be underestimated in the microarray experiment; the number of genes that are reciprocally antagonized by IFNγ and DHT is therefore likely to be greater. To test this functional interaction in vitro, we defined a model in which this antagonism could be recapitulated with a transiently transfected MMTV reporter system that has been extensively used to study the transcriptional properties of androgens (19). We assayed for luciferase activity in either 22Rv1 or MDA-MB-453 transfected with a MMTV-Luc reporter in the presence or absence of DHT, IFNγ, or both. Consistent with our observations on endogenous genes, treatment with IFNγ did not significantly influence MMTV activity in the absence of DHT in either MDA-MB-453 or 22Rv1 cells. However, as observed in the microarray, treatment with IFNγ suppressed up to 60% of DHT-induced activity in MDA-MB-453 but not 22Rv1 cells (Fig. 1D). The difference of sensitivity to IFNγ between the two cell lines was not due to IFNγ-induced cell death as no effect of IFNγ on proliferation and survival could be detected (data not shown). Hence, IFNγ could modulate DHT-induced reporter activity but not basal transcriptional activation from the MMTV promoter only in MDA-MB-453 cells. Taken together these observations demonstrated that DHT and interferon modulate each others signaling on a subset of endogenous genes as well as in the context of a reporter system.

The down-regulation of IFN-induced transcriptional activation did not appear to result from DHT down-regulating the genes that control the IFNγ signal transduction pathways as DHT had no effect on IFN-mediated up-regulation of genes involved in transduction of interferon action like Stat1 or IRF1 (Fig. 1B). The oligoadenylate synthase-RNase L signaling pathway is regulated by IFNs and mediates part of their antiviral activities (20). Mutations of RNase L have been linked to familial PCa (21). Examination of the RNase L sequence revealed the presence of two LXXL motifs at positions 187 and 583. This motif has been shown to mediate interaction with members of the nuclear hormone receptor family (24), suggesting a potential interaction between RNase L and the androgen receptor that would provide a mechanism for the reciprocal modulation of the two pathways. Testing for interaction between the two proteins by GST pull-down experiments revealed that AR and RNase L can interact with each other. As shown in Fig. 2B, under conditions where little or no interaction was detected between RNase L and GST, an AR-ΔNAD-GST fusion protein could readily pull-down RNase L. Addition of 100 nM DHT during the incubation and washes did not affect the interaction suggesting that, at least in vitro, the interaction is not hormone dependent (Fig. 2B). Introduction of the R462Q and D541E mutations into RNase L (mRNase L) did not affect interaction with AR (Fig. 2B, right panel). To identify regions of RNase L that are important for interaction, amino and carboxyl-terminal deletions mutants were assayed in GST pull-down experiments. Deletion of up to 400 NH2-terminal amino acids did not significantly alter AR–RNase L interaction (data not shown). In contrast, deletion of the last carboxy-terminal 170 amino acid residues greatly diminished the interaction (lane B, Fig. 2C). Further deletion of 170 amino acid residues almost completely abolished interaction with AR (lane C, Fig. 2C) indicating that both the kinase-like domain and the RNase domain of the protein are important for interaction with AR. Interestingly, under conditions where interaction with AR-ΔNAD could be readily detected, RNase L did not interact with the glucocorticoid receptor ligand-binding domain GR-LBD, in the presence or absence of dexamethasone (Fig. 2C, lane A, far right), suggesting that interaction is restricted to AR. To confirm this interaction in a cellular context, we performed a series of co-immunoprecipitation experiments on MDA-MB-453 extracts. Whereas a control IgG antibody could not immunoprecipitate AR or RNase L (data not shown), we found that RNase L co-immunoprecipitates with an AR-specific antibody (Fig. 2D). In contrast to GST pull-down data, treatment of MDA-MB-453 cells with 100 nM DHT for 60 min resulted in increased interaction between AR and RNase L compared with Me2SO-treated extracts. Co-immunoprecipitation using a RNase L-specific antibody gave similar data (Fig. 2D). Taken together, these
data demonstrate that in MDA-MB-453, AR and RNase L interact in a ligand-dependent manner.

In light of IFNγ effect on DHT-induced gene transcription (Fig. 1, B and D), and of the interaction between RNase L and AR, we tested the effect of IFNγ on DHT-mediated induction of the MMTV reporter system in the context of overexpressed wild type or mutated RNase L. Co-transfection of wild type RNase L expression vector had no effect on DHT induction of the MMTV promoter in the absence of IFNγ, not even at higher RNase L-to-reporter ratios (Fig. 3A). However, in cells treated with 500 units/ml IFNγ, overexpression of RNase L led to a marked attenuation of DHT-mediated transcriptional activation. This effect was dose-dependent, as increasing amounts of cotransfected RNase L expression resulted in further reduction of DHT-induced activation by IFNγ (Fig. 3B). In contrast, similar expression level of mRNase L did not affect DHT induction of the MMTV promoter activity even in the presence of IFNγ (Fig. 3, C and D). Under similar conditions, overexpressed wild type RNase L did not confer the MMTV promoter sensitivity to IFNγ in 22RV1 cells (data not shown). The lack of effect of IFNγ in the context of overexpressed mutated RNase L in MDA-MB-453 cells (Fig. 3D) or of overexpressed wild type RNase L in 22RV1 cells (data not shown) suggests that RNase L activity might not be the sole determinant in this mechanism, since mutant R462Q activity is not totally abrogated (7). It is possible that the reduced activity of the R462Q mutant compounded to its reduced ability to dimerize contributes to the lack of IFNγ effect. Alternatively the D541E mutation could affect RNase L properties in the context of its heterodimerization with AR. The MMTV promoter is also responsive to GR agonists. Consistent with the lack of RNase L binding to GST-GR(LBD) (Fig. 2C), co-transfection of either wild type or mutated RNase L did not affect induction of the MMTV promoter by dexamethasone (Fig. 3E). Taken together, these data suggest that the level of RNase L activity plays an important and selective role in IFNγ-mediated down-regulation of AR signaling.

22RV1 cells have high level expression of AR and are androgen-independent for growth (25, 26). In the light of our findings, it is conceivable that increased AR activity might account for 22RV1 lack of response to IFNs. To test for this hypothesis, we down-regulated AR expression in 22RV1 cells using siRNA and monitored the effect of IFNγ on genes that had previously been found in our microarray analysis to be highly induced by IFNγ in MDA-MB-453 cells but only marginally induced in 22RV1 cells. Transfection of AR-specific siRNA in 22RV1 cells resulted in a specific and pronounced down-regulation of AR mRNA (supplemental Fig. 1) and protein (Fig. 3F, inset) that persisted for at least 48 h. Consistent with our microarray data, Stat1 was marginally activated by IFNγ in naïve 22RV1 cells or cells transfected with nonspecific siRNA (NS siRNA) (Fig. 3F). Strikingly, down-regulation of AR expression was sufficient to restore responsiveness to IFNγ (Fig. 3F). Similar results were obtained with GBP1 genes (data not shown). These data demonstrate that, in the absence of DHT, activated AR can suppress response to interferon in PCA cells.

The significance of interferon signaling to prostate cancer progression is not entirely understood. RNase L acts as a tumor suppressor that, in the presence of interferons, stimulates apoptosis in prostate cells (6, 7). Furthermore, many attempts to characterize pathways involved in PCA tumor progression have
implicated IFN signaling. Proteomic analysis revealed a down-regulation of IFN-inducible genes in cancer versus normal epithelial cells derived from the same patient (27). Likewise, Shou et al. (28) have shown that the acquisition of tumorigenic characteristics by BPH1 cells is accompanied by a down-regulation of IFN-responsive genes. An earlier report also showed that transition of LNCaP cells to an androgen-independent state is associated with a reduction of AR expression and a concomitant up-regulation of IFN-induced genes (29).

Significantly, many of the IFN-inducible genes shown by us to be sensitive to AR signaling, like IFIT IFI35, GBP2, and MX1, where found to be dysregulated in all three studies. In light of our data, we suggest that androgen signaling contributes to the down-regulation of IFN-induced genes during the transition from benign to malignant phenotype. Our data support a model whereby, under normal circumstances, androgens and interferons balance the effect of each other (Fig. 3G, top). Activation of AR, by ligands, cytokines, or mutation, directly affects cell survival through AR signaling but also reduce tumor suppression by IFN signaling (Fig. 3G, middle). Although a contribution of other branches of the interferons signaling, like MX1 or PKR, cannot be excluded on the basis of our observations, we show evidences that AR-IFN cross-talk branches of the interferons signaling, like MX1 or PKR, cannot be excluded. We have shown that AR could recruit RNase L to nascent RNAs at the promoter of DHT-mediated genes. The molecular mechanisms by which interaction between AR and RNase L could mediate a reciprocal antagonism between DHT and IFN are not clear. Since both proteins function as a homodimer, it is conceivable that 2'-5' A- or DHT-mediated heterodimerization could affect both RNase L and AR activities. This notion is supported by our observation that overexpression of the dimerization-deficient R462Q mutant could not antagonize DHT activity in our system. Alternatively, AR could recruit RNase L to nascent RNAs at the promoter of DHT-regulated genes thereby targeting them for IFN-stimulated and specific degradation. RNase L has been shown to attenuate IFN response by destabilizing mRNAs (30). In this context DHT-dependent interaction with AR could enhance this activity. The cross-talk we described here can account for the earlier tumor onset observed in a subset of HPCI patients (23). On a broader context, our findings document how the dysregulation of distinct signaling pathways can synergize and promote cancer cells survival and tumor progression.

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