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THE ARYL HYDROCARBON NUCLEAR TRANSLOCATOR ALTERS CD30-MEDIATED NF- κ B-DEPENDENT TRANSCRIPTION

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Abstract

Expression and signaling of CD30, a tumor necrosis factor (TNF) receptor family member, is upregulated in numerous lymphoid-derived neoplasias, most notably anaplastic large cell lymphoma (ALCL) and Hodgkin's lymphoma (HL). To gain insight into the mechanism of CD30 signaling, we used an affinity purification strategy that led to the identification of the aryl hydrocarbon receptor nuclear translocator (ARNT) as a CD30 interacting protein that modulated the activity of the RelB subunit of the transcription factor nuclear factor (NF)- κ B. ALCL cells deficient in ARNT exhibited defects in RelB recruitment to NF- κ B-responsive promoters whereas RelA recruitment to the same sites was potentiated, resulting in augmented expression of these NF- κ B-responsive genes. These findings indicate that ARNT functions in concert with RelB in a CD30-induced negative feedback mechanism.

The activation of the transcription factor NF- κ B as a consequence of signaling from the tumor necrosis factor (TNF) receptor family member CD30 accounts for many of the physiological phenomena regulated by CD30 (1,2). For example, CD30 signaling contributes to tumorigenesis of Hodgkin's lymphoma cells (HL) by increasing NF- κ B activity (3,4). TNF receptor associated factor (TRAF) proteins are important for CD30-mediated NF- κ B activation (5–8), but other proteins may also participate in regulation of CD30 signaling. Furthermore, aspects of NF- κ B regulation remain enigmatic, especially regarding the mechanisms that control NF- κ B transactivation at the chromatin level (9). Therefore, in an attempt to identify signaling intermediates between CD30 and NF- κ B activity, we conducted a biochemical affinity screen (10,11) in Karpas 299, a cell line exhibiting inducible CD30 signaling that was derived from a patient with anaplastic large cell lymphoma (ALCL) (12). Analysis of the biochemically purified products revealed the aryl hydrocarbon receptor nuclear translocator (ARNT; also known as hypoxia-inducible factor 1- β) as a CD30 interacting protein (Fig. 1, S1). ARNT is a transcription factor that is integral for the regulation of xenobiotic and hypoxic responses (13–15).

We cloned the coding sequence of ARNT from Karpas 299 cells and used it to derive nested deletions of ARNT (Fig. S1C). We tested three major regions of ARNT, the basic-helix-loop-helix (bHLH) domain, the Period-ARNT-Single minded (PAS) domain (a protein-protein interface), and the transactivation domain, for their ability to bind the cytoplasmic region of

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CD30. Precipitation analysis of the different ARNT constructs with a tagged form of CD30, in which the cytoplasmic domain of CD30 was fused downstream of glutathione-S-transferase (GST), confirmed that ARNT interacted with CD30 through the PAS and transactivation domains (Fig. 1A). Thus, at least two regions of ARNT are responsible for its binding to the cytoplasmic tail of CD30, and other regions of ARNT appear to negatively regulate this binding (Fig. 1A).

To explore the spatial and temporal co-localization of CD30 and ARNT, we surface labeled Karpas 299 cells, expressing either GFP or ARNT-GFP, with a CD30-phytoerythrin (PE) conjugated antibody. Confocal microscopy showed that CD30 was predominantly localized to the plasma membrane three hours after stimulation with anti-CD30-PE (Fig. 1B and S2), whereas ARNT was localized to the nucleus, as expected, and no co-localization was detected with CD30 at the plasma membrane. In contrast, twenty-four hours after stimulation, all of the originally surface labeled CD30 was found in the cytoplasm, consistent with a previous report (16). ARNT-GFP was also detected in the cytoplasm at this later time point where it co-localized with internalized CD30. A panel of classical NF- κ B responsive genes showed increased transcription when CD30 was labeled with anti-CD30-PE to a level comparable to that in cells stimulated for 10 min with CD30 ligand (CD30L; Fig. 1C and 2A), ensuring that anti-CD30-PE induces CD30 signaling. Thus, the association of ARNT with CD30 appears to occur in the cytoplasm late in CD30 signaling.

The interaction of ARNT with CD30 was greatly impaired if the last 36 amino acids of CD30 were deleted (Fig. 1C). The sequences for TRAF binding are within these 36 amino acids of CD30 and the ability to recruit the TRAF proteins is required for full NF- κ B activation (8), suggesting that ARNT might influence CD30-mediated NF- κ B activity. We investigated this possibility with two short interfering RNA (siRNA) oligonucleotides that suppress ARNT expression in Karpas 299 cells. After ARNT suppression, Karpas 299 cells were exposed to either a 10 min CD30 stimulation by the physiological CD30 ligand (CD30L; Fig. 2A) or a continuous suboptimal dose of agonistic antibody to the type 2 TNF receptor (TNFR2; Fig. 2B), a TNF receptor family member closely related to CD30. Real-time PCR analysis was used to measure receptor-dependent transcriptional activity from the endogenous NF- κ B responsive genes intercellular adhesion molecule-1 (*ICAM1*), RelB (*RELB*) and inhibitor of κ B alpha (*I κ B α* ; *NFKBIA*) (Fig. 2). In cells in which ARNT was suppressed the CD30-induced increase in mRNA abundance from NF- κ B responsive genes was 2- to 3-fold greater than that in control cells (Fig 2A). A similar trend was observed for a TNFR2 signal in the absence of ARNT. While basal levels of NF- κ B activity were increased prior to a TNFR2 signal in cells where ARNT was suppressed there was a significant augmentation in NF- κ B activation in ARNT-suppressed cells after a TNFR2 signal, although we cannot rule out the possibility of a TNFR2-independent role for ARNT in NF- κ B regulation (Fig. 2B). The TNFR2-dependent activation of NF- κ B exhibited a characteristic biphasic activation as previously described (17,18).

Stimulation of CD30 had minimal effect on basal transcription from genes regulated by ARNT, including cytochrome P450 (*CYP1A1*) and the facilitated glucose transporter (*SLC2A*) (Fig. 2), suggesting that the observed increases in NF- κ B induced transcription appear to result from loss of ARNT and not a change in ARNT-responsive gene transcription. Furthermore, for genes considered ARNT responsive, the loss of ARNT resulted in decreased expression, even under normoxic conditions, indicating that the observed augmentation in NF- κ B transactivation was not the result of a general increase in transcription.

To uncover the regulatory control point of ARNT in the NF- κ B pathway, nuclear extracts from control-suppressed and ARNT-suppressed Karpas 299 cells were analyzed by an electrophoretic mobility shift assay (EMSA). Suppression of ARNT resulted in an altered migration pattern of the NF- κ B-DNA probe complex after CD30 stimulation (Fig. 3A),

suggesting that ARNT is a component of the NF- κ B complex or directs the binding of one or more NF- κ B subunits, or both. To address this, each NF- κ B subunit was fused with GST, individually co-expressed with HA-tagged ARNT in HEK293 cells and precipitated with glutathione-sepharose. ARNT did not interact with GST-RelA or GST-p50 but was precipitated with GST-RelB, and to a lesser extent associated with GST-c-Rel and GST-p52 (Fig. 3B). The interaction of RelB with ARNT was confirmed by co-immunoprecipitation of the endogenous proteins from Karpas 299 nuclear extracts following a 10-min treatment with CD30L. The association of RelB with ARNT in the nucleus correlated with CD30 stimulation (Fig 3C). We propose that ARNT may bind to RelB and direct the DNA binding of RelB containing complexes during a CD30 signal.

The significance of the RelB-ARNT interaction was further examined through studies in HEK293 cells by expressing different combinations of ARNT, RelA, RelB and p50. Nuclear extracts isolated from these cells and analyzed by EMSA showed that binding of ectopically expressed ARNT to a NF- κ B specific probe was not detected and ARNT had little effect on the binding of RelA homodimers or RelA-p50 heterodimers (Fig. 3D). RelB did not bind to the NF- κ B probe when expressed alone, as expected, and the addition of ARNT did not change the amount of RelB homodimer binding. The expression of ARNT with RelB-p50 heterodimers did result in a slight enhancement of binding (Fig. 3D), and co-expressing ARNT with RelB-p50 at two different concentrations revealed a dose-dependent enhancement of RelB-p50 DNA binding (Fig. 3E). These data raise the possibility that the presence of ARNT facilitated the binding of RelB containing complexes to DNA.

RelB has both transcriptional promoting and repressing activity depending on the signaling context or cell type (19–22). Our observations (Fig. 2 and 3) suggest that RelB is a repressor of CD30-mediated canonical NF- κ B responsive genes. We thus examined the binding pattern of endogenous NF- κ B subunits to the promoters of the *RELB* and *NFKBIA* (*I κ B α*) genes by chromatin immunoprecipitation (ChIP). RelB was present on the promoters before a CD30 signal was initiated and was subsequently replaced by RelA three hours after CD30 stimulation (Fig. 4A–D, siControl). HDAC1, a co-repressor, bound to the *RELB* promoter in a pattern similar to that of the RelB subunit, suggesting that RelB might recruit HDAC1 to effectively repress RelA-p50 activity (Fig 4A and fig. S4A). To test whether the augmentation of NF- κ B transcription observed in the absence of ARNT (Fig. 2) was a direct result of decreased RelB binding to NF- κ B responsive promoters, promoter binding was analyzed in ARNT-suppressed Karpas 299 cells. When ARNT was depleted the amount of RelB bound to promoters prior to CD30 stimulation was decreased. Furthermore, binding of RelB to the indicated promoters was diminished at later time points (Fig. 4A and C; siARNT) when, in control cells, CD30-dependent transcription would normally begin to decline (Fig. 2). The decrease in RelB-promoter binding, in cells where ARNT was suppressed, was not a function of destabilized RelB protein or defective RelB translocation to the nucleus (Fig. S3B). While RelB promoter binding was decreased in ARNT-suppressed cells, RelA appeared at the promoters sooner and in higher amounts. This was concomitant with enhanced recruitment of the co-activator Bcl-3 (Fig. 4A and C; S4A and B) whereas, in the case of the *RELB* promoter, recruitment of HDAC1 was diminished (Fig. 4A, S4A). These data support the observed increase in transcription of NF- κ B responsive genes in CD30-stimulated cells lacking ARNT (Fig. 2) and reveal the role of ARNT as a possible cofactor of RelB repressive function.

How NF- κ B selectively binds and is regulated at κ B sites in target promoters is an area of intense interest but remains incompletely understood (23–27). We observe a repressive function for RelB in a TNF receptor pathway and provide evidence for a role of ARNT in regulation of DNA binding by RelB containing complexes (Fig 5). These findings provide a possible mechanistic basis for how ARNT might be redirected from TNF receptor superfamily

signaling by a xenobiotic or hypoxic insult towards a transcriptional role, thus resulting in altered NF- κ B activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Association of ARNT with CD30. **(A)** Two regions of ARNT mediate the interaction with CD30. HEK293 cells were transfected with plasmids for the expression of combinations of GST-CD30 and HA-tagged versions of the mutants depicted in Supplementary Fig. S1C. CD30 pulldowns (PD) were performed with glutathione (GSH)-sepharose and the eluates were probed for ARNT (HA) and CD30 (GST). **(B)** Localization of CD30 with ARNT in the cytoplasm late in CD30 signaling. Karpas 299 cells expressing ARNT-GFP were labeled with anti-CD30-PE and analyzed by confocal microscopy at 3 and 24 hours. **(C)** Anti-CD30-PE is an agonistic antibody that stimulates CD30-mediated NF- κ B activation. Karpas 299 cells were treated as indicated for 3 hours. Total RNA was isolated and subjected to reverse transcription

followed by analysis by real-time PCR. All samples were normalized to control unstimulated cells. **(D)** CD30 associates with ARNT through the cytoplasmic TRAF binding domain. HEK293 cells were transfected with plasmids for the expression of ARNT-HA and either GST, GST-CD30, or GST-CD30 Δ 36. Lysates were precipitated with glutathione-sepharose and the eluates were probed for ARNT (HA) and CD30 (GST). *A*, *B*, and *D* are representative of one individual experiment that was repeated 3 times. *C* represents two independent experiments performed in triplicate.

Fig. 2.

Augmented CD30-mediated activation of NF- κ B after suppression of ARNT. Control siRNA (siControl) or ARNT siRNA (siARNT) oligonucleotides were introduced into Karpas 299 cells followed by a 10-minute exposure to (A) CD30L or (B) continuous exposure to a suboptimal concentration of agonistic TNFR2 antibody. Cells were collected at the times indicated, total RNA was isolated and subjected to reverse transcription followed by quantitative real-time PCR analysis for the indicated genes. All samples were normalized to control unstimulated cells. The results represent the mean $\pm$ SD of three independent experiments performed in triplicate. Statistical analyses were performed using a two-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc analysis. (A) The expression of the NF- κ B responsive genes *ICAM1*, *RELB*, and *NFKBIA* (*I κ B α*) was increased in ARNT-suppressed cells compared to that in siControl cells at each time point after CD30 stimulation (* p <0.01). (B) ARNT suppression increased basal and TNFR-induced expression of *ICAM1* and *NFKBIA* at

each time point (* $p < 0.01$). Basal expression of *RELB* was not increased, but expression was increased at 3 to 9 hours after TNFR2 stimulation in cells in which ARNT was depleted (* $p < 0.01$). In ARNT-depleted cells, expression of *ICAM1* and *RELB* was increased at 3 and 9 hours after TNFR2 stimulation as compared to that in ARNT-suppressed cells at time 0 (# $p < 0.01$). Similarly, expression of *NFKBIA* in ARNT-suppressed cells was increased at 3, 9 and 12 hours after TNFR2 stimulation as compared to that in ARNT-suppressed cells at time 0 (# $p < 0.01$). (A) and (B) The basal expression of the ARNT responsive genes *CYP1A1* and *SLC2A1* was significantly reduced in the absence of ARNT (* $p < 0.01$). To confirm suppression of ARNT, cDNA was analyzed (*ARNT* graph; * $p < 0.01$) and lysates from unstimulated samples were probed for ARNT and β -actin.

Fig. 3.

ARNT as a NF-κB-associated co-regulator. **(A)** ARNT modulates NF-κB DNA binding potential. Karpas 299 cells were transfected with either control siRNA (siControl) or ARNT siRNA (siARNT). Nuclear extracts were probed for ARNT to ensure suppression and analyzed by EMSA. The *asterisk* denotes altered migration of the NF-κB-DNA complex. **(B)** and **(C)** Binding of ARNT to the NF-κB subunit, RelB. **(B)** Lysates from HEK293 cells transfected with the indicated expression plasmids were precipitated with glutathione (GSH)-sepharose. Inputs were probed for ARNT (HA) or the NF-κB subunits (GST) and the elutions were probed for ARNT. **(C)** Nuclear extracts from Karpas 299 cells were prepared at the indicated times following a 10-min CD30L exposure. Immunoprecipitations were performed with a RelB or

control antibody and eluates were probed for ARNT. **(D)** Effect of ARNT on NF- κ B subunit binding to DNA. Nuclear extracts prepared from HEK293 cells transfected with the indicated plasmids were analyzed by EMSA. The binding of RelA-p50 complexes was 1.07-fold greater in the presence of ARNT whereas the binding of RelB-p50 complexes was 1.3-fold greater in the presence of ARNT as determined by densitometry analysis. **(E)** Effect of ARNT on RelB-p50 binding to DNA. RelB and p50 were expressed with control vector, 0.5 μ g or 1 μ g of vector encoding ARNT in HEK293 cells. Nuclear extracts were prepared and analyzed by EMSA. The expression of ARNT increased the binding of RelB-p50 complexes by 1.5- (0.5 μ g of ARNT) and 2-fold (1 μ g of ARNT) as determined by densitometry analysis. All experiments were performed at least 3 times, and representative data are shown.

Fig. 4.

Effects of ARNT on RelB DNA binding. (A–D) Decreased RelB DNA binding in cells depleted of ARNT. Karpas 299 cells were transfected with control siRNA (siControl) or ARNT siRNA (siARNT) followed by CD30 stimulation. At the indicated time points the cells were collected and ChIP was performed with the specified antibodies. *RELB* (A, B) and *NFKBIA* (C, D) promoter binding was analyzed using conventional and quantitative PCR. Data (A) and (C) are representative of one experiment that was performed twice. Samples for quantitative PCR (B) and (D) were normalized to the applicable input samples and the results represent percent input of two experiments performed in triplicate.

Fig 5.

Proposed model of ARNT regulation of CD30-mediated NF-κB activity. Before CD30 stimulation RelB is present at classical RelA-p50 regulated target promoters. An initial CD30 signal results in RelA-p50 translocation to the nucleus, which replaces the RelB complex, and transcription ensues. As the CD30 signal progresses, RelB translocates to the nucleus where it associates with ARNT, enhancing the binding of RelB to DNA, and negatively regulating RelA-p50 complexes. Furthermore, ARNT associates with the TRAF binding region of internalized CD30, which may assist in the negative feedback loop of CD30 signaling by blocking TRAF binding.