

Research article

DEK binding to class II MHC Y-box sequences is gene- and allele-specificBarbara S Adams,¹ Hyuk C Cha,¹ Joanne Cleary,² Haiying Tan,¹ Hongling Wang,¹ Kajal Sitwala,² David M Markovitz²¹Department of Pediatrics, Division of Pediatric Rheumatology, University of Michigan School of Medicine, Ann Arbor, MI, USA²Department of Internal Medicine, University of Michigan School of Medicine, Ann Arbor, MI, USA

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Arthritis Res Ther 2003, **5**:R226-R233 (DOI 10.1186/ar774)© 2003 Adams *et al.*, licensee BioMed Central Ltd (Print ISSN 1478-6354; Online ISSN 1478-6362). This is an Open Access article: verbatim copying and redistribution of this article are permitted in all media for any purpose, provided this notice is preserved along with the article's original URL.**Abstract**

Using electrophoretic mobility shift assays, we examined sequence-specific binding of DEK, a potential autoantigen in juvenile rheumatoid arthritis, to conserved Y-box regulatory sequences in class II MHC gene promoters. Nuclear extracts from several cell lines of different phenotypes contained sequence-specific binding activity recognizing *DRA*, *DQA1*0101*, and *DQA1*0501* Y-box sequences. Participation of both DEK and NF-Y in the *DQA1* Y-box binding complex was confirmed by 'supershifting' with anti-DEK and anti-NF-Y antibodies. Recombinant DEK also bound specifically to the *DQA1*0101* Y box and to the polymorphic *DQA1*0501* Y box,

but not to the consensus *DRA* Y box. Measurement of the apparent dissociation constants demonstrated a two- to fivefold difference in DEK binding to the *DQA1* Y-box sequence in comparison with other class II MHC Y-box sequences. Residues that are crucial for DEK binding to the *DQA1*0101* Y box were identified by DNase I footprinting. The specific characteristics of DEK binding to these related sequences suggests a potential role for DEK in differential regulation of class II MHC expression, and thus in the pathogenesis of juvenile rheumatoid arthritis and other autoimmune diseases.

Keywords: DEK, genetic polymorphism, *HLA-DQA1*, *HLA-DRA*, juvenile rheumatoid arthritis**Introduction**

Although juvenile rheumatoid arthritis (JRA) is the most common cause of disability in children, its etiology is unknown. Immune dysregulation appears to play a key pathogenic role, as circulating autoantibodies are common in patients with certain JRA clinical subtypes [1–7]. Two recent studies have shown a highly significant association between early-onset pauciarticular JRA and circulating antibodies to the 43-kDa nuclear protein DEK [8,9]. Although circulating DEK antibodies have subsequently been found in children and adults with other autoimmune diseases [10,11], these two studies did reveal that children with JRA are significantly more likely to have anti-DEK antibodies than are children without rheumatic disease. Children with pauciarticular-onset JRA were also significantly more likely to have anti-DEK antibodies than were

children with polyarticular-onset or systemic-onset JRA or other rheumatic diseases. Among children with pauciarticular JRA, DEK autoantibodies were significantly more common in those with JRA-associated uveitis than in those without eye disease [8,9]. DEK reactivity was also found to be strongly associated with onset of any JRA subtype before age 6 years [8].

DEK is a nuclear protein that is not structurally related to any known family of proteins [12,13]. Although it may also participate in DNA replication and RNA processing [14,15], we have identified DEK as a DNA-binding protein that recognizes the TG-rich peri-ets (pets) regulatory element in the human immunodeficiency virus type 2 (HIV-2) enhancer [16]. The pets site is important in mediating HIV-2 enhancer stimulation in activated T cells and

monocytes [17–19], suggesting that DEK may play an immunomodulatory role as it participates in transcriptional activation through this and related sites.

Observed sequence similarity between the DEK-binding site in HIV-2 and the highly conserved Y-box regulatory element in MHC class II gene promoters pointed to the Y box as one possible related site. NF-Y binding to the MHC class II gene Y box anchors a complex assembly of nuclear proteins that occupies several regulatory elements over a great distance [20–22]. In the *DQA1* promoter Y box, a reverse CCAAT motif with a partially overlapping TG-rich sequence shares sequence identity with the HIV-2 DEK-binding site at 7 of 10 positions (Fig. 1). In the *DQA1*0501* allele, which is highly associated with predisposition to autoimmune disease [23–27], the Y box contains a single-nucleotide polymorphism that reduces sequence identity to 6 of 10 positions. We hypothesized that DEK could bind in a sequence-specific manner to the Y-box motifs in the promoter regions of several class II MHC genes, and that gene- and allele-specific Y-box polymorphisms could affect DEK binding activity. In this study, we examine the characteristics of DEK binding to the Y-box sequences of *DQA1*0101*, *DQA1*0501*, *DRA*, *DQB*, and *DRB*. We also confirm participation of DEK with NF-Y in the *DQA1* Y-box binding complex and localize specific DEK binding within this sequence. As the Y-box promoter element is crucial to the regulation of MHC class II gene expression, sequence-specific binding to this motif indicates a potential role for DEK in modulating normal and abnormal immune response.

Materials and methods

Cell culture and preparation of nuclear extracts

Cultured cell lines were grown and harvested and nuclear extracts were prepared from resting cells as previously described [28,29].

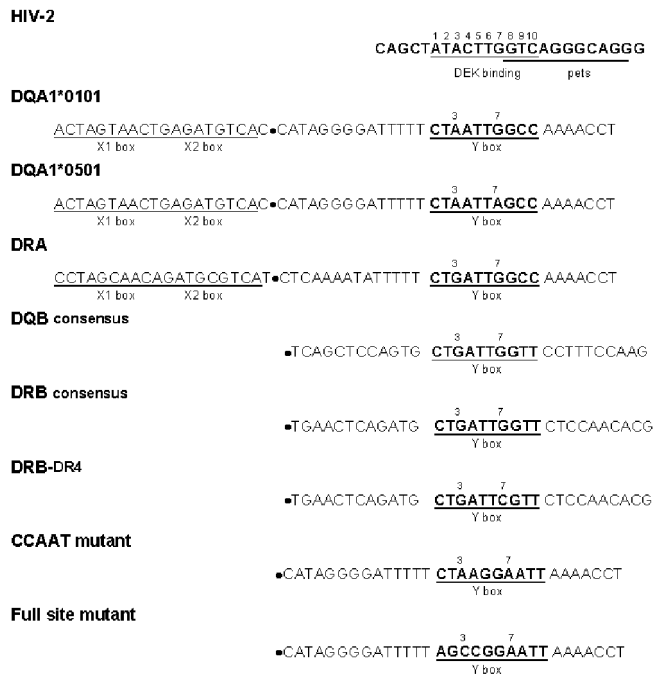
Preparation of partially purified recombinant DEK protein

Construction of the poly-histidine-tagged DEK bacterial expression vector is described elsewhere [16]. Full-length DEK or antisense DEK was prepared from cultures grown from individual colonies to log phase, induced with 1 mM isopropyl thiogalactose, and harvested by centrifugation after 4 hours. Recombinant protein was purified from bacterial lysates in accordance with the published method for the QIAexpress system (Qiagen, Valencia, CA, USA) with variations in Buffers B and D as noted in Supplementary material. Procedures were carried out at 4°C; dialyzed recombinant DEK protein (rDEK) was stored at –80°C.

Preparation of FLAG-DEK

A FLAG-tagged DEK adenoviral vector constructed by the University of Michigan Vector Core was used to transduce T98G cells (ATCC) by incubation for 48 hours before harvesting for immunoprecipitation. FLAG-DEK was

Figure 1



EMSA probes and competitors: HIV-2 DEK-binding site, class II MHC Y-box motifs (*DQA1*, *DRA*, *DQB*, and *DRB*), and related sequences. Probes and competitors include only sequences 3' of the ● symbol. X boxes are shown to provide a broader context for the Y-box regulatory element. EMSA = electrophoretic mobility shift assay.

immunoprecipitated using anti-FLAG resin (Sigma-Aldrich, St Louis, MO, USA) in accordance with the manufacturer's instructions and was eluted by competition with peptide containing three FLAG recognition epitopes.

Electrophoretic mobility shift assays (EMSAs)

EMSAs were carried out as previously described [30], using 0.1–0.25 ng of radiolabeled oligonucleotide probe (2.5×10^4 counts per minute) per 15 µl binding reaction and 5 µg of nuclear extract (except as noted) or <1 µg of rDEK. For competition EMSAs, unlabeled double-stranded oligonucleotide was added to reaction mixtures before the radiolabeled probe. For antibody supershift of binding complexes, 1 µl anti-NF-YA antibody (gift of JP-Y Ting) or 1 µl high-titer anti-DEK human serum (gift of W Szer [9]) or 2–3 µl control human serum was added to the binding reaction, and the mixture was incubated on ice for 2 hours before the probe was added.

Sequence of oligonucleotide probes and competitors

See Fig. 1.

Measurement of apparent dissociation constants using EMSA

The ³²P end-labeled oligonucleotide probe (5 nM) was incubated with immunoprecipitation-purified FLAG-rDEK in a

range of concentrations from one tenth to 10 times the estimated $K_{d[app]}$ (apparent dissociation constant) as described elsewhere [31]. Protein-bound DNA was separated from free probe as for EMSA in 1 × TBE (Tris-borate-EDTA buffer: Tris 89 mM, borate 89 mM, EDTA 2 mM). The dried gel was exposed to a phosphor screen overnight, and the bands were quantified using a Molecular Dynamics Storm 840 Phosphorimager with ImageQuant Software. The data were fit via nonlinear least-squares regression to the single-site binding isotherm:

$$\% \text{ free DNA} = K_{d[app]} / (K_{d[app]} + [\text{protein}]).$$

From this equation, the apparent K_d corresponds to the protein concentration at which half of the DNA is bound [31].

DNase I protection assay

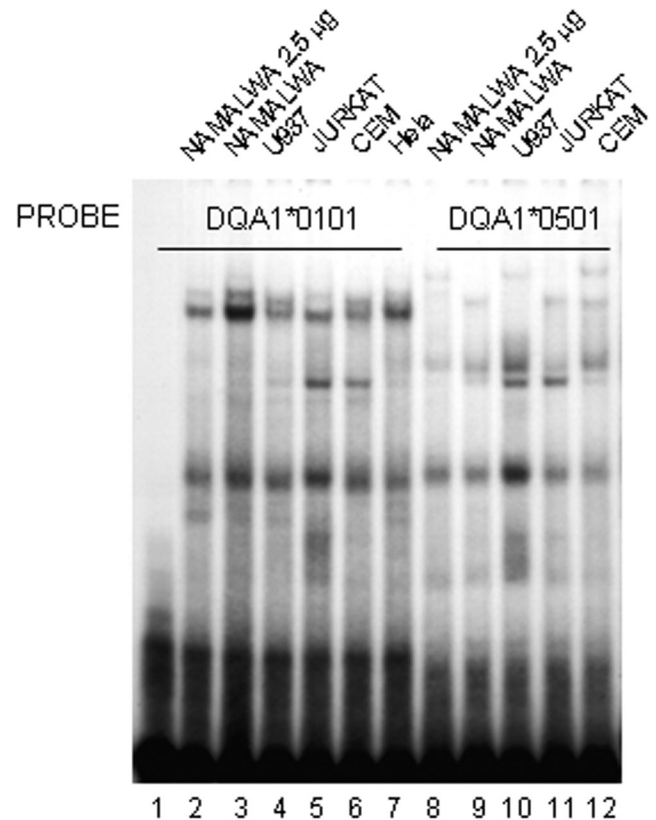
The 148-bp probe included *DQA1*0101* promoter sequence from -53 to -200. PCR primer (200 ng) for probe sequence was end-labeled with $^{32}P\gamma$ -ATP and T4 polynucleotide kinase (New England Biolabs, Beverly, MA, USA), and was column purified. Labeled antisense primer (200 ng) and 200 ng unlabeled sense strand primer (or vice versa) were used in each 50.5- μ l PCR reaction, with 0.2 mM dNTP, 1.5 mM $MgCl_2$, 5 μ l 10X PCR Buffer (Invitrogen, Carlsbad, CA, USA), and 1 μ g Namalwa genomic DNA. *Taq* polymerase (Invitrogen) was added at 80°C after a 94°C 'hot start,' initiating 35 cycles of PCR: 94°C for 45 s, 55°C for 30 s, and 72°C for 90 s, with final extension at 72°C for 10 min. PCR products were purified with a High Pure PCR Product Purification Kit (Roche Applied Science, Indianapolis, IN, USA) and then used at 2.5×10^4 CPM/2 μ l. DNase I digestion reaction and footprinting gel followed published techniques [16].

Results

Y-box binding activity in nuclear extracts is gene- and allele-specific

The similarity of the *DQA1*0101* Y-box sequence to the HIV-2 DEK-binding site (see Fig. 1) first led us to investigate whether DEK in nuclear extracts binds to MHC class II Y-box regulatory elements *in vitro*. With a double-stranded oligonucleotide probe containing the *HLA-DQA1*0101* Y-box sequence, EMSAs revealed similar binding activity in nuclear extracts from B lymphoid (Namalwa), T lymphoid (Jurkat, CEM), monocytoid (U937), and HeLa cell lines (Fig. 2), and in nuclear extracts from SKW 6.4 (B lymphoid), KG-1 (T lymphoid), and HL-60 (monocytoid) cell lines (not shown). Electrophoretic patterns with a probe corresponding to the *DQA1*0501* Y-box sequence, which diverges from the *DQA1*0101* Y-box sequence by a single base pair within the highly conserved reverse CCAAT sequence, appear to vary with the cell type, and binding appears weaker than with the *DQA1*0101* probe (see Fig. 2).

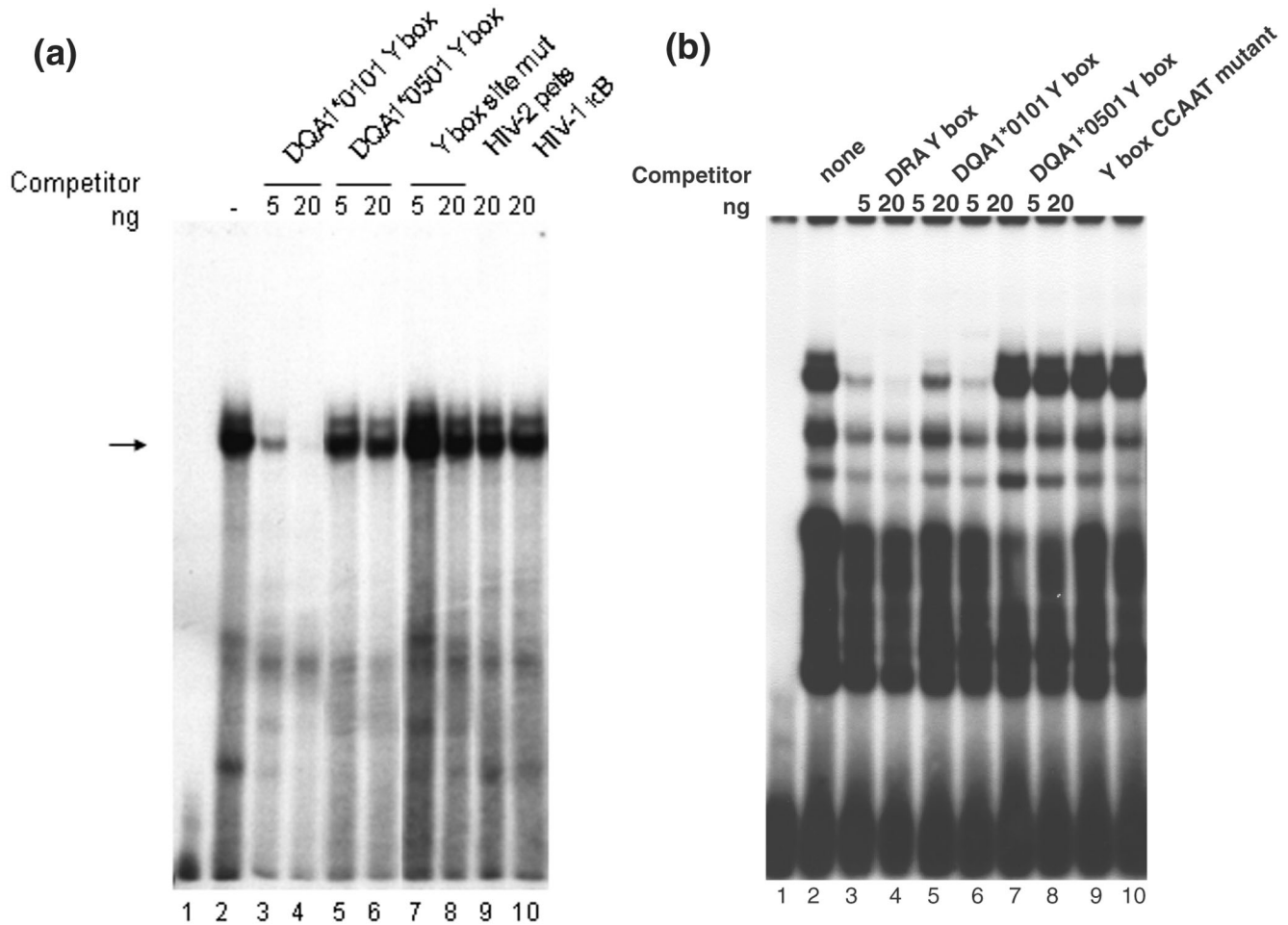
Figure 2



Nuclear extracts from several cultured cell lines show *DQA1* Y-box-specific binding activity. Oligonucleotide probes containing the *DQA1*0101* Y-box sequence (lanes 1-7) or *DQA1*0501* polymorphic Y-box sequence (lanes 8-12) bind protein in nuclear extracts prepared from resting cells of the indicated cultured cell lines. Lane 1, control without nuclear extract; lanes 2 and 8, 2.5 μ g of protein; lanes 3-7 and 9-12, 5 μ g of protein.

Competition EMSAs demonstrated sequence-specificity of the *DQA1*0101* Y-box binding activity (Fig. 3a). Unlabeled *DQA1*0101* oligonucleotides successfully competed for strong binding activity seen in nuclear extracts from the Namalwa B cell line (Fig. 3a, lanes 3 and 4), whereas unlabeled *DQA1*0501* Y-box sequence competed less well (Fig. 3a, lanes 5 and 6). There was no competition when the Y-box reverse CCAAT motif was mutated at all five positions (Fig. 3a, lanes 7 and 8), nor with the unrelated HIV-1 κ B sequence (lane 10). DEK has been shown to bind to the HIV-2 pets site [16], but oligonucleotides containing this sequence also failed to compete significantly (lane 9), suggesting that factors other than DEK play a role in determining the specificity of this complex. In particular, NF-Y is the predominant nuclear factor binding to the Y box [21], and the HIV-2 pets site does not contain the reverse CCAAT sequence required for NF-Y binding.

Figure 3



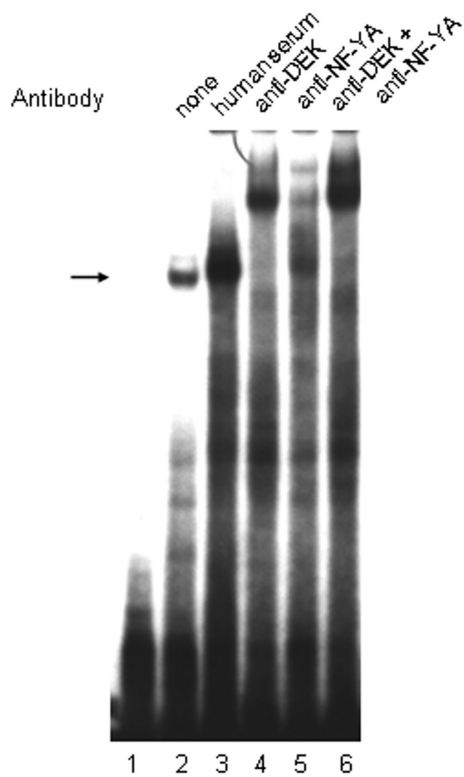
Namalwa cell nuclear protein(s) bind to *DQA1* and *DRA* Y-box elements. 5 μ g of Namalwa cell nuclear extract (lanes 2–10) and unlabeled oligonucleotide competitors were added to the binding reaction before addition of the radiolabeled *DQA1**0101 probe (a) or the radiolabeled *DRA* probe (b) to define the sequence specificity of nuclear protein binding. In both (a) and (b), lane 1 contains no protein. See Figure 1 for site mutant and CCAAT mutant sequences. *pets* = *peri-ets*.

The consensus (*HLA-DRA*) Y-box sequence differs from the *HLA-DQA1**0101 Y box by a single nucleotide immediately 5' of the reverse CCAAT pentamer; this single-nucleotide polymorphism significantly changes the electrophoretic pattern (Fig. 3b) in comparison with that seen with the *HLA-DQA1**0101 probe. *HLA-DQA1**0101 oligonucleotide also competes poorly with the consensus Y-box probe for binding (Fig. 3b, lanes 5–6). Divergence of the *HLA-DQA1**0501 Y-box sequence at one position within the required NF-Y binding site reduces its ability to compete with the *DRA* Y-box probe (Fig. 3b, lanes 7–8) as much as does mutation of all five nucleotides within the CCAAT sequence (Fig. 3b, lanes 9–10).

Both DEK and NF-Y participate in the *HLA-DQA1* Y-box binding complex

Participation of both DEK and NF-Y in the *DQA1* Y-box binding complex was confirmed by using high-titer anti-DEK antiserum (gift of W Szer [9]) and monoclonal antibody to the NF-YA subunit of the Y-box binding factor (gift of JP-Y Ting) to further retard the mobility of the *DQA1* Y-box binding complex ('supershifting') (Fig. 4, lanes 3–4). Preincubation of Namalwa cell nuclear extracts with antibodies to NF-YA also retards the binding complex (Fig. 4, lane 5), leaving a residual band (arrow) which can be attributed to distinct DEK binding activity. Preincubation with both antibodies results in further retardation of the binding

Figure 4



DEK and NF-Y participation in the *DQA1*0101* Y-box binding complex demonstrated by 'supershift' assay. Namalwa nuclear extract (5 µg) was preincubated with the following antibody reagents before addition of radiolabeled probe containing *DQA1*0101* Y-box sequence: lane 2, no antibody reagent; lane 3, 2 µl normal human serum; lane 4, 1 µl high-titer anti-DEK human antiserum; lane 5, 1 µl anti-NF-YA (subunit) monoclonal antibody; lane 6, 1 µl of anti-DEK plus 1 µl of anti-NF-YA monoclonal antibody. Lane 1 contains probe without nuclear extract or antibody reagent.

complex to form a doublet (Fig. 4, lane 6). The same electrophoretic patterns were seen after preincubation of nuclear extracts from the CEM T lymphocytoid cell line with anti-DEK and anti-NF-YA antibodies (data not shown).

Recombinant DEK protein binds in a sequence-specific manner to the *DQA1* Y box but not to the *DRA* (consensus) Y box

Having established that DEK and NF-Y in nuclear extracts participate in the *HLA-DQA1*0101* binding complex, we used recombinant full-length DEK (rDEK) to determine whether it alone can bind to Y-box motifs and to examine how gene- and allele-specific sequence polymorphisms alter its binding. The *HLA-DQA1*0101* Y-box sequence does bind rDEK specifically, with unlabeled probe sequence competing successfully for binding (Fig. 5a, lanes 4–6). In contrast, despite its identity at 9 of 10 positions, unlabeled consensus (*DRA*) Y-box sequence (Fig. 5a, lanes 7–8) competes very poorly against the *HLA-DQA1*0101*

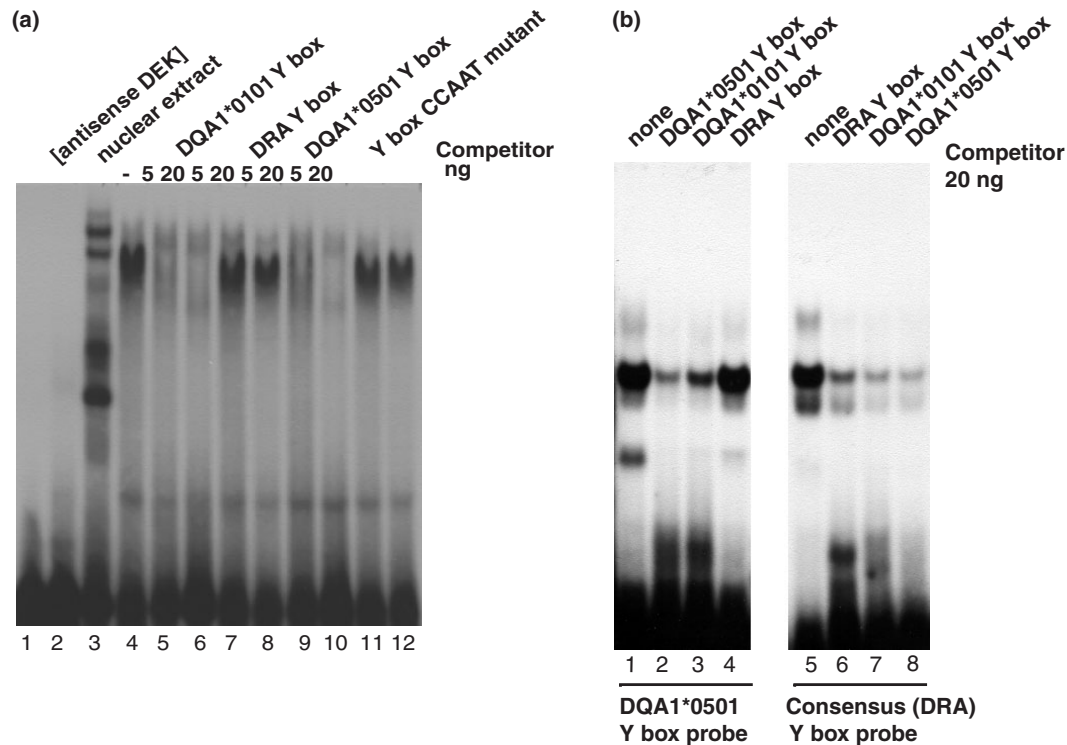
Y-box probe. *DQA1*0501* Y-box sequence (Fig. 5a, lanes 9–10) competes only slightly less well than does *DQA1*0101*, indicating that a change in the residue at position 7 does not prevent rDEK binding. Mutation of the reverse CCAAT sequence, however, eliminates effective competition for binding to the *DQA1*0101* Y-box sequence (Fig. 5a, lanes 11–12). Taken together, these results indicate that substitution of adenine at position 3 in the *DQA1* Y box for guanine in the consensus Y-box sequence significantly strengthens DEK binding, as do bases at positions 5 and 6 in the reverse CCAAT sequence.

Further characterization of rDEK binding to the polymorphic *DQA1*0501* Y-box sequence and to the consensus (*HLA-DRA*) Y-box sequence establishes a relative hierarchy of DEK binding activity (Fig. 5b). With the *DQA1*0501* probe, unlabelled *DQA1*0101* sequence (Fig. 5b, lane 3) competed less well than did unlabelled probe. *DRA* Y box sequence (Fig. 5b, lane 4) and CCAAT mutant sequence (not shown) did not compete for binding. Inability of the *HLA-DRA* Y-box sequence to compete with the *DQA1*0501* probe again indicates the relative importance of the adenine residue immediately 5' to the reverse CCAAT for DEK binding. With the *DRA* Y-box sequence as probe (Fig. 5b, lanes 6–10), the unlabelled probe sequence competes less well than do any of the other Y-box sequences, suggesting that rDEK alone binds to this sequence nonspecifically and with low affinity.

Quantitative assessment of rDEK binding to related DQ- and DR- Y-box sequences

Quantification of the apparent dissociation constant ($K_{d[app]}$) for rDEK binding to related class II MHC Y-box motifs validates the relative hierarchy of DEK binding activity described above, and further emphasizes the contribution of gene-specific Y-box polymorphisms to DEK binding activity. As shown in Table 1, DEK binds more strongly to Y-box sequences in either *DQA1* allele than it does to any of the other *DR-* or *DQ-*related Y-box sequences. It binds least well to the *DRA* (consensus) Y-box motif, with a $K_{d[app]}$ that is approximately five times that for the *DQA1* sequences. The $K_{d[app]}$ for DEK binding to *DQB* is approximately four times that for the *DQA1* sequences, whereas the $K_{d[app]}$ for DEK binding to the *DRB* Y-box sequences, especially the *DRB* alleles associated with the DR4 haplotype, are intermediate between the two extremes. Once again, the A-to-G substitution at position 3 in the Y box appears to strengthen DEK binding to the *DQA1* Y box in comparison with the *DRA* Y box; allelic variation in the nucleotide at position 7 in the Y-box sequence (as in *DQA1*0501* and in the DR4-associated *DRB* Y-box sequence) may also mediate subtle differences in DEK binding to otherwise identical sites. Gene-specific sequence polymorphisms outside the Y box per se could also explain why dissociation constants differ where Y-box sequences are identical, as in the *DQB* and *DRB* consensus motifs.

Figure 5



Recombinant DEK protein (rDEK) binds in a sequence-specific manner to the *DQA1*0101* Y box, but not to the *DRA* (consensus) Y box. **(a)** 5 μ l of antisense rDEK (lane 2), 5 μ g of Namalwa cell nuclear extract (lane 3), or 5 μ l of partially purified recombinant DEK protein (rDEK) (lanes 4–12) was used in each EMSA binding reaction; rDEK was preincubated with the indicated unlabelled competitor before addition of the *DQA1*0101* Y-box probe. Lane 1 contains no protein. **(b)** The indicated unlabelled oligonucleotide competitors (20 ng) were added to the binding reaction containing 5 μ l of partially purified rDEK before addition of radiolabeled *DQA1*0501* probe (lanes 1–4), or radiolabeled *DRA* probe (lanes 5–8). PolyD(I-C) (10 ng) was added to each binding reaction as nonspecific competitor. EMSA = electrophoretic mobility shift assay.

Table 1

Apparent dissociation constant ($K_{d[app]}$) of rDEK binding to class II MHC Y-box sites

Sequence name	Y-box sequence	$K_{d[app]}^a$ (nM)
<i>DQA1*0101</i>	CTAATTGGCC	478 \pm 103
<i>DQA1*0501</i>	CTAATTAGCC	582 \pm 130
<i>DRA</i>	CTGATTGGCC	2480 \pm 519
<i>DQB</i> consensus	CTGATTGGTT	1977 \pm 93
<i>DRB</i> consensus	CTGATTGGTT	1266 \pm 274
<i>DRB-DR4</i>	CTGATTGGTT	1041 \pm 258

^aMean \pm standard error, based on at least three determinations.

Localization of DEK binding within the Y box

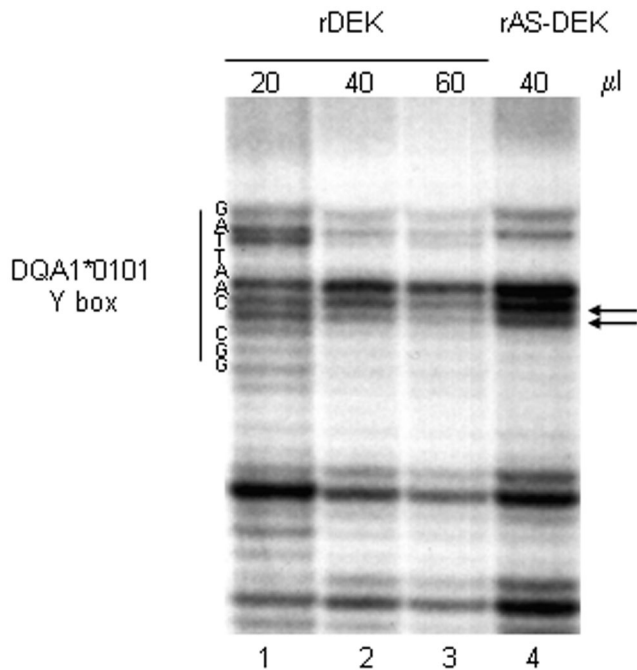
DNase I footprinting with recombinant protein further defined the physical interaction between DEK and the *HLA-DQA1*0101* Y-box element (Fig. 6). Using the non-coding strand as probe, consistent protection is seen over the G at position 7, which is polymorphic in the

*DQA1*0501* allele (A-for-G substitution) and in the DRB alleles associated with the DR4 haplotype (C-for-G substitution). Consistent protection is also seen at position 2, adjacent to the A-for-G substitution that diverges from the Y-box consensus sequence, although there is no protection of the divergent base itself at position 3. DNase I protection by recombinant DEK extends over the length of the Y-box sequence, including bases within the NF-Y binding site (reverse CCAAT sequence), again suggesting that the two proteins may interact.

Discussion

All antigen-presenting cells upregulate MHC class II transcription in response to immune stimulation. We have previously shown that activation of promyeloid cells causes dephosphorylation of DEK and diminished DEK binding to the HIV-2 long terminal repeat [16,17,32]. In this study, we show that rDEK can bind to the *DQA1* Y box, and that DEK in nuclear extracts participates in the *DQA1* Y-box binding complex *in vitro*. Thus, we propose a model in which intracellular signaling modulates the ability of DEK to bind DNA, causing alteration of MHC class II transcrip-

Figure 6



DNase I footprinting localizes rDEK binding to the *HLA-DQA1*0101* Y box. Partially purified rDEK (20–60 µl) was incubated with *DQA1*0101* probe (antisense strand). Antisense rDEK (40 µl) (prepared simultaneously with the rDEK protein) was used as a control. The Y-box sequence is shown on the left. Arrows denote consistent protection against DNase I digestion in multiple assays. rAS-DEK = recombinant anti-sense DEK protein; rDEK = recombinant DEK protein.

tion. Transient transfection experiments in cultured cell lines have not proved useful in examining this model, for DEK is highly expressed in most cells of hematopoietic lineage, and further overexpression has resulted in apparently nonspecific downregulation of transcriptional activity (as might be predicted from [14]). For this reason, we are currently pursuing other experimental approaches.

The *DQA1*0501* promoter region (QAP 4.1), in which a single-base-pair polymorphism in the Y box significantly diminishes transcriptional activity [33], is a component of the so-called susceptibility haplotype for autoimmune disease. The *DQA1*0501* allele is strongly associated with early-onset pauciarticular JRA in Northern European populations [23,24] and with increased risk for juvenile dermatomyositis [25] and Sjögren's syndrome with high autoantibody production [26,27]. In our proposed model, aberrant class II MHC regulation could result from altered DEK binding and/or interaction with NF-Y, and aberrant class II expression may alter or enhance reactivity against DEK-derived or other self peptides. The C-terminal region of DEK, which contains the putative DNA-binding domain,

appears to be most antigenic [34] (and K Sitwala and DM Markovitz, unpublished observations), raising the possibility that altered DNA binding may expose other masked epitopes. Development of antibodies to DEK could even be a primary event in the pathogenesis of JRA, with disruption of nuclear events due to penetration of anti-DEK antibodies into living cells [35]. It remains to be determined whether anti-DEK antibodies are directly involved in the pathogenesis of autoimmune disease, or if they result from generally enhanced immunoreactivity.

Conclusion

Three specific findings in this report support a potential role for DEK as a transcriptional modulator of MHC class II expression. One is that DEK binds to the *HLA-DQA1* Y box in a sequence-specific manner. Another is that NF-Y and DEK both participate in the *HLA-DQA1* Y-box binding complex, which coordinates DQ protein expression. Finally, DEK binds differentially to specific Y-box sequences found in *HLA-DQA1*0101* and **0501* alleles) and *HLA-DRA*, consistent with observations of *DQA1* gene-specific cell-surface expression [36] and allele-specific promoter activity [33]. The specificity of rDEK binding to these and other, related Y-box sequences (including *HLA-DQB* and *HLA-DRB* alleles associated with the DR4 haplotype) may correlate with a predisposition to autoimmune disease seen with certain HLA haplotypes.

Competing interests

None declared.

Acknowledgements

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Supplementary material

Buffer B (6 M urea, 0.1 M NaH₂PO₄, 10 mM Tris-l, 0.1 M KCl), used for cell lysis and protein elution, was prepared with the addition of imidazole (final concentration 25 mM) and urea (final concentration approximately 8 M). Qiagen Ni-NTA Superflow resin was equilibrated with modified buffer B before being combined with the cell lysate. The cell lysate-resin combination was incubated at 4°C for 1.5 hours. Purified protein was eluted from the column with buffer B containing 100–250 mM imidazole. The presence and size of rDEK bands were verified by western blotting. Protein in positive elution fractions was refolded to native structure by serial dialysis against buffer D (20 mM HEPES, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 20% glycerol) containing stepwise decreasing concentrations of urea (6 M for 2 hours, 4 M for 2 hours, 2 M for 2 hours, no urea for 12 hours).