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Efficient Repression of Endogenous Major Histocompatibility Complex Class II Expression through Dominant Negative CIITA Mutants Isolated by a Functional Selection Strategy

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Major histocompatibility complex class II (MHC-II) molecules present peptide antigens to CD4-positive T cells and are of critical importance for the immune response. The MHC-II transactivator CIITA is essential for all aspects of MHC-II gene expression examined so far and thus constitutes a master regulator of MHC-II expression. In this study, we generated and analyzed mutant CIITA molecules which are able to suppress endogenous MHC-II expression in a dominant negative manner for both constitutive and inducible MHC-II expression. Dominant negative CIITA mutants were generated via specific restriction sites and by functional selection from a library of random N-terminal CIITA deletions. This functional selection strategy was very effective, leading to strong dominant negative CIITA mutants in which the N-terminal acidic and proline/serine/threonine-rich regions were completely deleted. Dominant negative activity is dependent on an intact C terminus. Efficient repression of endogenous MHC-II mRNA levels was quantified by RNase protection analysis. The quantitative effects of various dominant negative CIITA mutants on mRNA expression levels of the different MHC-II isotypes are very similar. The optimized dominant negative CIITA mutants isolated by functional selection should be useful for in vivo repression of MHC-II expression.

Major histocompatibility complex class II (MHC-II) molecules play a central role in both humoral and cellular immune responses by presenting peptide antigens to CD4-positive T cells. Quantitative differences in MHC-II expression directly influence the magnitude of the T-cell response (20). MHC-II molecules are αβ transmembrane heterodimers. Three isotypes, HLA-DR, -DQ, and -DP, can be distinguished in humans. MHC-II molecules show a complex pattern of expression that is regulated mainly at the level of transcription. Constitutive MHC-II expression is confined to professional antigen-presenting cells such as dendritic cells, macrophages, and mature B cells. Expression of MHC-II can be induced on a large number of other cell types by various stimuli, among which gamma interferon (IFN-γ) is most prominent (4, 14, 25).

Analysis of the mechanisms controlling MHC-II expression has been greatly facilitated by the existence of cell lines with genetic defects in essential factors controlling transcription of MHC-II genes (16, 25). These cell lines are derived mainly from patients suffering from primary MHC-II deficiency, also called the bare lymphocyte syndrome (BLS). Certain cell lines generated in vitro display the same phenotype. MHC-II deficiency is genetically heterogeneous, and the existence of several complementation groups has revealed the existence of several regulatory factors which are essential for MHC-II expression (25). Three of these factors, CIITA, RFX5, and RFXAP, have been identified by genetic and biochemical approaches (13, 36, 38). RFX5 and RFXAP are subunits of the RFX complex, which binds to the X-box DNA element of MHC-II promoters (13, 31, 36). CIITA, the first MHC-II regulatory factor shown to be defective in MHC-II deficiency (38), has been identified as a master regulator of both constitutive and inducible MHC-II expression (32, 35, 38, 39). CIITA is itself differentially expressed, and we have shown that induction of MHC-II by IFN-γ is mediated via the induction of CIITA, which is an obligatory intermediate in the signalling cascade (39). CIITA has been shown to be both necessary and sufficient to induce MHC-II expression in numerous cell types. It activates MHC-II transcription via the proximal MHC-II promoters, and there is a direct positive correlation not only qualitatively but also quantitatively between the level of CIITA and MHC-II expression (28, 39).

CIITA is a large protein of 1,130 amino acids (aa) whose mode of action remains obscure. In addition to an ATP/GTP binding consensus sequence and two leucine-rich repeats near its C terminus, CIITA contains at its N-terminal end an acidic region and three proline/serine/threonine (P/S/T)-rich regions reminiscent of transcriptional activation domains (38). The acidic domain is able to act as a transcriptional activation domain when fused to heterologous DNA binding domains (33, 47). Furthermore, replacement of the acidic domain of CIITA by viral activation domains is sufficient to activate MHC-II transcription (37, 47). Since binding of CIITA to MHC-II promoters has not been demonstrated, these observations led to the hypothesis that CIITA may act as a transcriptional coactivator that contacts MHC-II promoter binding proteins through its C-terminal 800 aa (33, 38, 47).

Dominant negative mutants of wild-type proteins have been useful tools with which to study protein function (29). Dominant negative mutants of CIITA would be interesting for several reasons. Since a large part of the CIITA protein is not functionally characterized, and there is no direct biochemical assay for CIITA function, delimiting functional domains in CIITA via dominant negative mutants would contribute to the understanding of its mode of action and make it more amenable to biochemical studies. Strong dominant negative CIITA mutants would also be useful for in vivo studies of immune function through repression of MHC-II expression. Given the

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quantitative correlation between MHC-II expression and T-cell activation (20), the experimental downregulation of MHC-II expression via dominant negative CIITA mutants could be a valuable tool with which to evaluate the contribution of MHC-II expression in the immune response, for example, in animal models of autoimmune diseases and transplantation.

We report here the construction and analysis of a large number of CIITA mutants generated both by deleting specific regions and via functional selection from a library of randomly generated mutants. Fluorescence-activated cell sorting (FACS) analysis and quantitative analysis of endogenous MHC-II mRNA levels by RNase protection demonstrate a strong reduction of both constitutive and inducible MHC-II expression, affecting all MHC-II isotypes. By expression cloning, we have isolated several strong dominant negative CIITA mutants which may be sufficiently effective to be used in in vivo experiments.

MATERIALS AND METHODS

Cells and cell culture. The Epstein-Barr virus-positive Burkitt lymphoma cell lines Raji and RJ2.2.5 were a gift from R. S. Accolla. Raji and RJ2.2.5 cells were grown in RPMI 1640 (RPMI), and HeLa cells were grown in Dulbecco modified Eagle medium, both supplemented with 10% fetal calf serum (FCS; PAA Laboratories GmbH) and 1% penicillin-streptomycin-glutamine, at 37°C in 5% CO_2.

Vectors. Constructs were all generated in pBluescriptKS(+) (Stratagene) and then cloned into the expression vector EBO-76PL (35), EBS-PL, or KEBS-PL. For the construction of EBS-PL, the simian virus 40 (SV40) early enhancer/promoter controlling the cDNA expression cassette of EBO-76PL was replaced with the SRα promoter from pCDL-SRα-296 (41). The sequences of EBO-76PL and EBS-PL are available upon request. KEBS-PL was derived from EBS-PL by replacing an *Ase*I-*Hin*dIII fragment of EBS-PL containing the β-lactamase gene with a *Sal*I fragment containing a kanamycin resistance cassette (KnRTn*903*) derived from pUC4Kn (Pharmacia). All episomal expression vectors contain a hygromycin resistance gene under the control of an SV40 early promoter for antibiotic selection in mammalian cells.

CIITA deletion mutants. Deletion mutants were derived from NLS-CIITA, in which the second in-frame methionine, M(25), of the CIITA cDNA (38) is preceded by a nuclear localization signal (NLS)-containing sequence, MPKKKRKVH (where the NLS consensus is KKKRK). 3′ to the coding region, a synthetic oligonucleotide linker with multiple restriction sites and stop codons in all three reading frames was added. The predicted amino acid sequence of NLS-CIITA is therefore MPKKKRKVHM(25)-R(1130), with the numbers in parentheses indicating the CIITA amino acid positions as published elsewhere (38). An *Nde*I restriction site (CATATG) overlaps HM(25). From NLS-CIITA, N-terminal deletions were performed by digestion with *Nde*I and internal restriction sites in the CIITA cDNA. Where necessary, small double-stranded oligonucleotide adapters were added to maintain the reading frame. C-terminal deletions were obtained by digestion in the 3′ linker and internal CIITA restriction sites. For the D5 construct without the NLS, the NLS was removed via *Nde*I digestion and replaced by a 5′ oligonucleotide linker with a good Kozak consensus (aagcttgccactATG) sequence upstream of M(25). The deduced amino acid sequences of the constructs are as follows: NLS-D1, MPKKKRKVHMH(157)-R(1130); NLS-D4, MPKKKRKVHMS(218)-R(1130); NLS-D5, MP KKKRKV HMT(295)-R(1130); NLS-D6, MPKKKRKVHR(409)-R(1130); NLS-D7, MP-KKKRKVHRP(517)-R(1130); NLS-D8, MPKKKRKVHR(700)-R(1130); NLS-D9, MPKKKRKVHMP(974)-R(1130); NLS-10, MPKKKRKVHMH(157)-L(980)VK; NLS-D11, MPKKKRKVHMH(157)-A(790)S; NLS-D12, MPKKKRK VHMH(157)-P(699); NLS-D13, MP KKKRKVHMH(157)-L(644); and NLS-D14, MPKKKRKVHMH(157)-P(410).

The BLS-2 and BCH allele 2 CIITA cDNAs containing small in-frame deletions of aa 940 to 963 and 1079 to 1106, respectively, are derived from cDNA libraries of Epstein-Barr virus-transformed B-cell lines from the respective patients (5, 38). From these cDNAs the following constructs were generated: BLS-2, M(1)-Δ(940-963)-R(1130); NLS-BLS-2, MPKKKRKVHM(25)-Δ(940-963)-R(1130); NLS-BCH allele 2, MPKKKRKVHM(25)-Δ(1079-1106)-R(1130); NLS-D2, MPKKKRKVHMH(157)-Δ(940-963)-R(1130); and NLS-D3, MP KKKRKV HMH(157)-Δ(1079-1106)-R(1130). Nucleotide sequences are available on request.

Transfections. A total of 7×10^6 cells for analysis of single constructs, or 2×10^7 cells for libraries, were transfected by electroporation (38); transfected cells were selected with hygromycin B (Calbiochem) at 200 μg/ml and were cultivated under the constant presence of hygromycin. Selection was considered to be complete 2.5 weeks after transfection.

IFN-γ induction of HeLa cells. HeLa cell transfectants selected with hygromycin for 3 weeks were induced with 1,000 U of recombinant IFN-γ (Gibco BRL) per ml. FACS cell surface analysis was performed 3 days after exposure to IFN-γ.

FACS analysis. The following antibodies were used for staining the cells: HLA class II DR-specific 2.06 (10) (a gift from C. Epplen), DP-specific B7/21 (44) (a gift from N. Reinsmoen), DQ-specific Tü22 (48) (a gift from A. Ziegler), HLA class I-specific W6/32 (3), and CD45-specific mouse anti-human CD45-FITC (Serotec). Cell staining and FACS analysis were performed as described previously (38). Staining and analysis was performed on live cells. Cell viability before staining was greater than 95% as measured by trypan blue exclusion. Dead cells were excluded from the analysis by staining with propidium iodide and by their forward and sideways light-scattering properties.

RNase protection assay. Cytoplasmic RNA was isolated (45) from 3×10^7 hygromycin-selected transfected cells on the same day as or the day after FACS analysis. The DRA probe (15) linearized with *Hae*III protects 310 nucleotides of the DRA mRNA. The DRB, DQA, DQB, and HLA-A probes were generated by reverse transcription-PCR from Raji with gene-specific primers. The DRB, DQA, DQB, and HLA-A probes span 125, 114, 186, and 224 nucleotides of their respective mRNAs. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe protects 100 nucleotides when linearized with *Eco*RI. RNase protection assays (38) were performed with [^{32}P]UTP-labelled riboprobes. The specific activities of DRA and GAPDH probes were reduced fivefold compared to those of the other probes. Dried gels were first exposed to X-ray films, followed by PhosphorImager analysis and quantification with the ImageQuant program.

Western blot analysis. Polyclonal CIITA antibodies were produced by injection of recombinant CIITA into rabbits. CIITA cDNA with a His_6 tag preceding the second ATG [M(25)] was subcloned in the pT7-7 vector (40). This construct was transformed in *Escherichia coli* BL21(DE3)pLys5, and the tagged CIITA was produced by induction with isopropylthiogalactopyranoside. Recombinant CIITA present in the insoluble fraction of the bacterial pellet was resuspended in 6 M guanidine and purified on nickel agarose under denaturing conditions. The purified protein was injected into rabbits, and four boosts were performed.

Total protein was extracted from 10^7 cells (23); 50 μg of extract was separated on a sodium dodecyl sulfate–7.5% polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) by using a Trans-Blot semidry transfer cell (Bio-Rad) for 30 min at 15 V. Membranes were incubated with the serum diluted 1/2,000 in 1× blocking solution overnight at 4°C plus 1 h at room temperature and washed in 1× washing buffer (DIG buffer set; Boehringer Mannheim). Detection was performed with a peroxidase-labelled anti-rabbit antibody diluted 1/5,000 in the same buffers as mentioned above, followed by chemoluminescence detection (ECL kit; Amersham).

N-terminal deletion library of CIITA. (i) Library construction. In the NLS-CIITA construct MPKKKRKVHM(25)-R(1130), a double-stranded oligonucleotide was inserted into the *Nde*I restriction site (CATATG) encoding HM(25), leading to the sequence MPKKKRKgt*c*$^{(=V)}$*cat*$^{(=H)}$*acacttgg*caggctagcag*catatg*$^{(=M25)}$E(26)-R(1130). Letters in italics represent the newly generated *Bst*XI and *Nde*I restriction sites. This plasmid was digested with *Bst*XI and *Nde*I, and deletions were performed by using a double-stranded Nested Deletion kit (Pharmacia Biotech). Exonuclease III digestion was performed for 25 min at 37°C, withdrawing aliquots every 30 s. All fractions were pooled, and subsequent steps were carried out according to the manufacturer's instructions. The *Bst*XI 3′ overhang should fully protect the cat$^{(=H)}$ codon from S1 digestion. This was the case for 5 of 12 randomly picked clones; 3 of 12 were missing one nucleotide and 4 of 12 were missing two nucleotides. In certain clones selected from the library, this incomplete protection led to an amino acid exchange (see below).

(ii) Functional selection for downregulation of MHC-II expression. Cells were selected for MHC-II downregulation either with an anti-DR antibody plus complement or with magnetic beads. For the anti-DR-plus-complement selection, cells were washed once with phosphate-buffered saline–bovine serum albumin, stained (10^7 cells/ml) with anti-DR 2.06 antibody (10), washed with RPMI, and incubated for 30 min at 37°C, 10^7 cells/ml, in a 1:2 dilution of normal rabbit complement (Cedarlane) in RPMI. Complement had been previously preabsorbed on Raji cells three times 20 min at 4°C. Cells were washed three times in RPMI and resuspended in RPMI supplemented with 10% FCS, 100 μg of hygromycin per ml, and 10 μl of insulin-transferrin-sodium selenite (Sigma) per ml. Surviving cells were counted after 24 h. For negative selection with magnetic beads, cells were stained with anti-DR 2.06, washed three times with phosphate-buffered saline–bovine serum albumin, and incubated with goat anti-mouse immunoglobulin G-coated Dynabeads M-450 (Dynal), with an excess of 20 beads per cell; supernatant was washed in RPMI and resuspended in RPMI–10% FCS. Isolated cells were counted after 24 h.

The library was transfected for the first selection/sorting step into 2×10^7 Raji cells. After hygromycin selection, the library was negatively selected against MHC-II expression three times with anti-DR and complement. After amplification of the selected transfectants, the plasmids were rescued from 5×10^7 cells by alkaline lysis (38) and used to transform *E. coli* DH10B cells by electroporation; 5×10^4 independent colonies were harvested and amplified. Plasmid DNA from these clones was diluted 20-fold with empty EBS-PL vector DNA and transfected for the second selection/sorting step into 2×10^7 Raji cells. Two steps of negative selection were performed with anti-DR and complement, and a third step was performed with magnetic beads. A total of 3.3×10^4 *E. coli* colonies were rescued from 5×10^7 transfected Raji cells.

The 5′ fragments from DNA of the various plasmid pools and of individual clones were generated by digestion with a unique restriction site just 5′ of the

NLS and the internal restriction site *Kpn*I (yielding a fragment of 2,309 bp for a full-length cDNA) and analyzed by agarose gel electrophoresis. Of the 43 individual clones, 19 showed rearrangements in the vector part of the plasmid, and 13 of these represented a single aberrant plasmid species. This clone showed a weak dominant negative effect upon retransfection. The precise 5′ end of the CIITA cDNA in this clone could not be determined. The remaining 24 clones appeared normal; sequencing revealed that they were all in frame and were of seven different kinds. One clone of each kind was transfected individually into Raji cells, and its dominant negative effect was analyzed by FACS analysis of stable transfectants.

The amino acid sequences of the isolated clones are as follows: NLS-L102, MPKKKRKVHD(102)-R(1130); NLS-L155, MPKKKRKVHL(155)-R(1130); NLS-L169, MPKKKRKV*P*G(169)-R(1130); NLS-L294, MPKKKRKV*P*A(294)-R(1130); NLS-L312, MPKKKRKVHT(312)-R(1130); NLS-L321, MP KKKRK VHC(321)-R(1130); and NLS-L335, MPKKKRKVHP(335)-R(1130). Clone NLS-L356 is one of the clones which were randomly picked from the library before selection in order to analyze the quality of the library (see above). The amino acid sequence of NLS-L356 is MPKKKRKV*P*P(356)-R(1130). Nucleotide sequences are available upon request.

RESULTS

All mutant CIITA molecules discussed here were tested for repression of endogenous MHC-II expression in the strongly MHC-II-positive Burkitt lymphoma-derived B-cell line Raji. Thus, the mutant CIITA molecules have to exert their dominant negative effect in a completely wild-type situation and in a native chromatin context. We reasoned that this assay, albeit more difficult than a reporter gene-based approach, would lead to the identification of only the strongest dominant negative CIITA mutants. These are obviously the most interesting from the point of view of structure-function studies and are also of highest potential usefulness as transgenes for in vivo experiments.

The mutant CIITA cDNAs were expressed from a novel episomal expression vector, EBS-PL, in which the cDNAs are under the control of the strong SRα promoter (41). Due to the presence of the EBV origin of replication (Ori P), the vector is maintained episomally in the transfected cells. The high transfection efficiencies and relatively homogeneous expression patterns which can be achieved with this type of vector system (38) permitted the analysis to be carried out on bulk cultures which had been selected only for antibiotic resistance, thus allowing an objective comparison between different constructs. The episomal vector system was also chosen in order to be able to carry out an expression cloning approach with a library of N-terminal deletions of the CIITA cDNA.

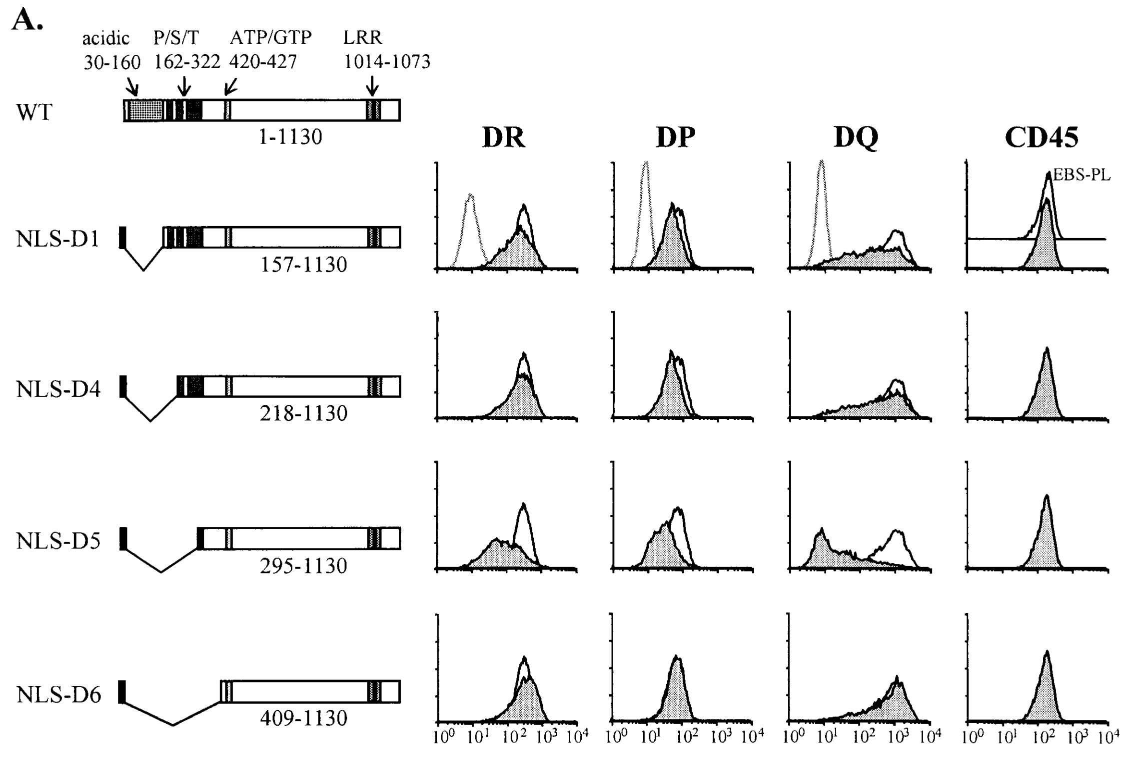
N-terminal deletions of CIITA lead to a dominant negative phenotype in a strongly MHC-II-positive B-cell line. Sequence analysis and functional studies had indicated that CIITA may act as a transcriptional coactivator with the N-terminal acidic and P/S/T-rich regions as activation domains (33, 38, 47). A number of N-terminal CIITA deletions were therefore constructed via specific restriction sites and tested for their ability to act as dominant negative proteins. We had established in pilot experiments that a CIITA construct with an SV40-derived N-terminal NLS fused to the second in-frame ATG codon of CIITA (amino acid position 25) had the same transactivating capacity as the wild-type construct (data not shown). The mutants were therefore expressed as NLS fusion proteins. After transfection into Raji and selection with hygromycin, the antibiotic-resistant bulk cultures were analyzed for cell surface HLA-DR, -DP and -DQ expression by flow cytometry (Fig. 1 and 2).

The CIITA mutant with the smallest N-terminal deletion, NLS-D1 (aa 157 to 1130), in which only the acidic region is deleted, clearly repressed all three isotypes, albeit the effect on DP was weak (Fig. 1A). In general, the dominant negative effect was most obvious on the HLA-DQ expression by FACS analysis but was consistently also observed for HLA-DR and -DP. A construct with a slightly larger deletion, also removing the first P/S/T-rich segment, NLS-D4 (aa 218 to 1130), was similar to NLS-D1 in strength. In contrast, the NLS-D5 construct (aa 295 to 1130), with an N-terminal deletion which leaves only the last 28 aa of the third P/S/T domain, leads to a very severe reduction of HLA-DR and -DP cell surface expression and an almost complete extinction of HLA-DQ expression (Fig. 1A). The relative strengths of the different constructs were reproduced consistently in repeated experiments. Further N-terminal deletions extending beyond NLS-D5 (NLS-D6, aa 409 to 1130, NLS-D7, NLS-D8, NLS-D9) completely abolished the dominant negative phenotype (Fig. 1A and 2 and data not shown). The dominant negative phenotype of NLS-D5 did not depend on the addition of the N-terminal NLS, since a construct without the NLS (D5) had the same capacity to repress MHC-II expression (Fig. 1C). The specificity of the dominant negative effect of the CIITA mutants was examined by cell surface analysis of MHC class I and CD45 molecules. No effect of the dominant negative CIITA constructs on these markers was observed (Fig. 1 and 4 and data not shown). The observed reduction of MHC-II expression was dependent on the presence of mutated CIITA constructs, since transfection of wild-type CIITA cDNA led to a mild overexpression of MHC-II molecules in Raji cells (data not shown).

We had shown earlier that small in-frame deletions near the very C-terminal end of CIITA were responsible for the MHC-II deficiency in two BLS patients (5, 38). Neither of the two patient-derived CIITA cDNAs containing only these C-terminal deletions (BLS-2, Δ940–963; BCH allele 2, Δ1079–1106) showed a dominant negative phenotype (Fig. 2 and data not shown). To examine the dependence of the dominant negative phenotype of NLS-D1 on the C-terminal part of the protein, we combined these two small internal C-terminal deletions, as well as five additional C-terminal truncations ranging from amino acid positions 410 to 980, with NLS-D1. None of these constructs showed a significant inhibition of MHC-II expression in Raji cells, demonstrating that not only the transactivation function but also the dominant negative phenotype of CIITA is dependent on an intact C terminus (Fig. 1b and 2 and data not shown).

Repression of IFN-γ-induced MHC-II expression by dominant negative CIITA constructs. We had shown earlier that IFN-γ-induced MHC-II expression is mediated by the induction of CIITA (39). We therefore wanted to verify whether the dominant negative mutants of CIITA also had the ability to repress IFN-γ-mediated MHC-II expression in HeLa cells (Fig. 3). The efficiency of IFN-γ induction was monitored by the analysis of MHC-I induction (data not shown). Induction of HLA-DR by IFN-γ was severely repressed by the CIITA mutants (Fig. 3). The relative strengths of the different mutants are the same as observed in the B-cell line, with NLS-D5 and D5 again having the strongest effect. Induction of HLA-DR was almost completely abolished by NLS-D5. These experiments show that repression of inducible MHC-II expression by dominant negative CIITA constructs is efficient. Furthermore, they indicate that the dominant negative effect of the CIITA mutants is not dependent on factors that are specific for B cells.

To investigate further the strength of the dominant negative phenotype of NLS-D5, this construct was cloned into the EBO-76PL expression vector (38). The SV40 early promoter in EBO-76PL is considerably less active than the SRα promoter (41). The resulting expression construct, EBO-NLS-D5, remains capable of exerting a strong repression on HLA-DR expression in HeLa cells (Fig. 3). The same construct is also capable of inducing a weaker but clearly detectable reduction


A.
acidic
30-160
P/S/T
162-322
ATP/GTP
420-427
LRR
1014-1073
WT
1-1130
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CD45
EBS-PL
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157-1130
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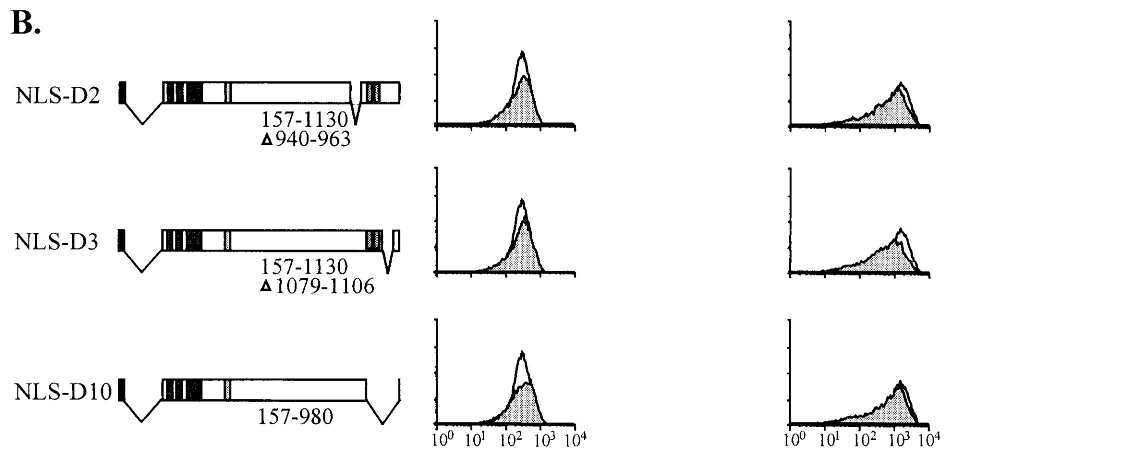
B.
NLS-D2
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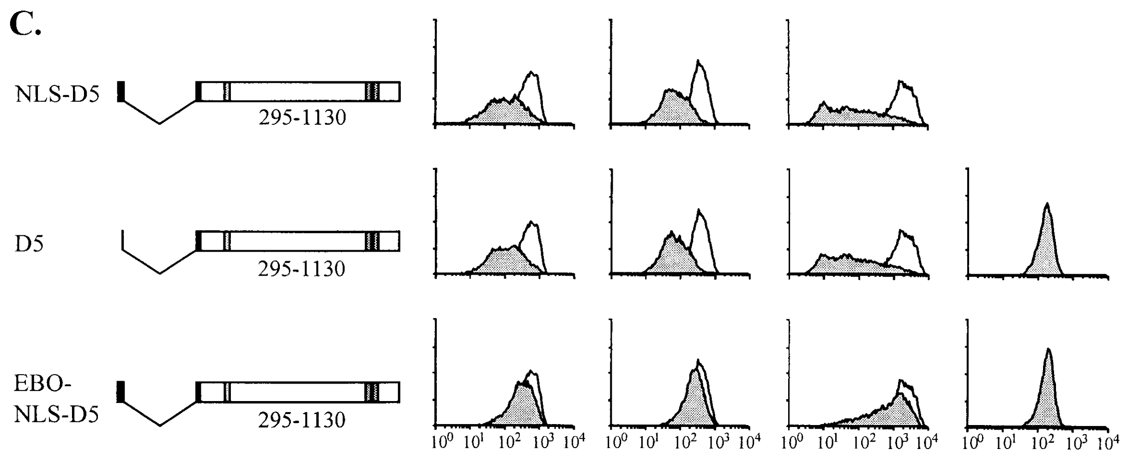
C.
NLS-D5
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D5
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FIG. 1. Dominant negative phenotypes of N-terminally deleted CIITA molecules. The different CIITA constructs are schematically represented on the left. Positions of the acidic and P/S/T domains, the ATP/GTP binding consensus, and the leucine-rich repeats (LRR) are indicated. Numbers indicate the N- and C-terminal amino acid positions of the mutant CIITA constructs. NLS indicates an N-terminal nuclear localization signal. Flow cytometry analysis of the stably transfected constructs in Raji is shown on the right. If not indicated otherwise, all constructs were expressed in EBS-PL. The different cell surface molecules analyzed are indicated above the FACS profiles. The filled profiles correspond to Raji transfected with the dominant negative constructs in EBS-PL; the open profiles represent a transfection of Raji with empty EBS-PL vector. In the first row of FACS profiles, the open profiles on the left correspond to the MHC-II-negative, CIITA-deficient RJ2.2.5 cell line. (A) Analysis of N-terminal CIITA deletions. (B) Double deletion combining NLS-D1 N-terminal and various C-terminal deletions. Internal deletions are indicated by Δ. (C) Comparison of NLS-D5, D5, and EBO-NLS-D5. Note that NLS-D5 shown in panels A and C results from two independent transfections. WT, wild type.

in Raji, particularly of HLA-DQ (Fig. 1c). The fact that a significant reduction in MHC-II expression is observed even at the low expression levels obtained with the EBO-76PL vector indicates that NLS-D5 has a strong dominant negative phenotype.

Reduction of MHC-II mRNA expression through dominant negative CIITA mutants. The effect of dominant negative CI ITA mutants on steady-state MHC-II mRNA levels was next analyzed (Fig. 4A). Cytoplasmic RNA was isolated from the various Raji transfectants, and HLA-DR and -DQ mRNA expression was analyzed by RNase protection. The results obtained on the mRNA level show the same gradient of dominant negative strength for the different CIITA constructs as observed previously for cell surface expression of MHC-II molecules (Fig. 4). Quantification of the results by PhosphorImager analysis showed a range from modest to very severe reductions of MHC-II mRNA levels (Fig. 4B). In the case of NLS-D5, mRNA levels were reduced between 65 and 80%, depending on the MHC-II gene examined. Analysis of two independent transfections with NLS-D5 showed very similar results (Fig. 4).

The apparent difference in the reduction observed between DR and DQ at the level of cell surface expression (Fig. 1) motivated us to compare DRA, DRB, DQA, and DQB expression at the mRNA level. Unexpectedly, despite the considerable differences in initial steady-state mRNA levels, very similar relative levels of reduction were observed for all of the MHC-II mRNAs analyzed (Fig. 4B). Thus, the difference in repression observed between DR and DQ at the cell surface is not reflected at the mRNA level. Instead, the dominant negative CIITA mutants affect the different MHC-II isotypes to the same extent, indicating that CIITA is not only qualitatively but also quantitatively of equal importance for all MHC-II isotypes.

Western blot analysis of dominant negative CIITA mutants. Protein levels of endogenous CIITA and of the dominant negative CIITA mutants were compared by Western blot experiments. A polyclonal rabbit anti-CIITA antiserum was raised against the complete human CIITA protein expressed in *E. coli*. This CIITA antiserum recognizes a doublet of approximately 130 kDa in Raji extracts (Fig. 5, lanes 2 to 5). Specificity is demonstrated by the complete absence of a signal in an extract from RJ2.2.5 (Fig. 5, lane 1). Experiments with various recombinant CIITA constructs have indicated that the upper and lower bands of the doublet correspond to CIITA translated from the first or second AUG (data not shown). In Raji cells transfected with the NLS-D5 construct, an additional band of the expected size is observed (Fig. 5, lane 3). Although the wild-type and NLS-D5 bands are similar in intensity, Western blot experiments with recombinant full-length and truncated CIITA constructs have indicated that the full-length CIITA protein is recognized by the antiserum better than the truncated proteins are (data not shown). Taking this finding into account, we estimate that the EBS-NLS-D5 CIITA construct is overexpressed by a factor of approximately 10 to 20 compared to the wild-type protein.

In vivo selection of dominant negative CIITA mutants from a library of N-terminal deletions. We adopted an expression cloning strategy to isolate additional dominant negative CIITA mutants from a library of N-terminally deleted CIITA clones. These experiments were carried out for several reasons. First, we wanted to delimit as precisely as possible the extent of the N-terminal region(s) that can be deleted without abolishing the dominant negative phenotype. Second, we wanted to isolate the strongest possible dominant negative CIITA mutants for future experiments, such as the generation of transgenic mice. Third, we wanted to evaluate whether this approach might be suitable for the selection of novel types of dominant negative CIITA mutants, for example, from CIITA libraries containing random point mutations.

A library of 5′-deleted CIITA cDNAs was generated by exonuclease III deletion and cloned into a derivative of EBS-PL, KEBS-PL. In KEBS-PL, the β-lactamase gene of EBS-PL was replaced by a kanamycin resistance cassette, which facilitated the dilution strategy described below. Analysis of randomly picked clones from the unselected library revealed the presence of N-terminal deletions ranging from 52 to 792 aa, with a majority of the clones initiating between aa 200 and 400. As expected, one-third of the clones had retained a correct reading frame. A plasmid pool corresponding to 40,000 independent clones was transfected into Raji, and stable transfectants were selected with hygromycin. Negative immunoselection was carried out three times with the anti-DR monoclonal antibody 2.06 (10) and rabbit complement. Plasmids were rescued from the transfected cells by alkaline lysis and transfor-

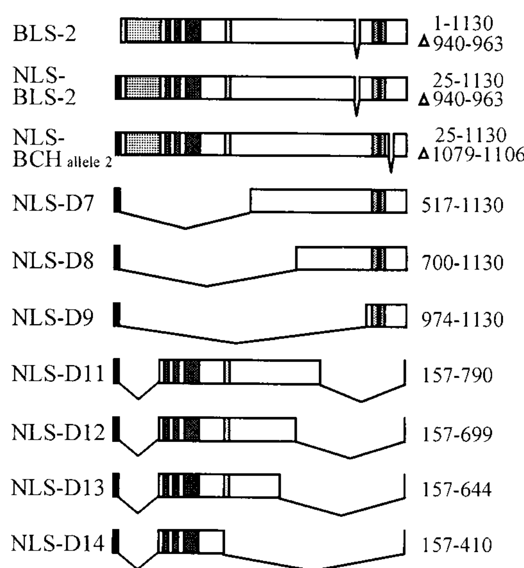


FIG. 2. CIITA mutants without dominant negative phenotype in Raji cells. All constructs were expressed in EBS-PL and analyzed as stable transfectants in Raji as shown in Fig. 1.

mation of *E. coli*. For retransfection, the rescued plasmid pool was diluted by a factor of 20 with EBS-PL (Amp^r) in order to eliminate nonfunctional coamplified passenger plasmids. After two rounds of negative selection with antibody and complement, a third round of negative selection was carried out with anti-DR and magnetic beads (Dynal). Comparison of the numbers of ampicillin- and kanamycin-resistant plasmids after the rescue indicated an enrichment of at least 100-fold for the second selection/sorting step (data not shown). Retransfection of the rescued, kanamycin-resistant plasmid population into Raji cells revealed a clear reduction of HLA-DR and -DQ expression (Fig. 6A).

The sizes of the 5′ ends of the CIITA inserts present in the plasmid pools rescued from the two selection/sorting steps and in the original input DNA were analyzed by gel electrophoresis. As shown in Fig. 6A, several prominent bands appear after two transfection/sorting steps, indicating a strong selection for plasmids which had either lacked only the acidic region or contained deletions extending to the C-terminal end of the third P/S/T domain. Analysis of 24 independent clones revealed the presence of seven different plasmid species. All clones were found to be in frame. Following transfection into Raji, six of the seven types repressed MHC-II expression. This result indicated that the dilution strategy had been very effective in eliminating nonfunctional plasmids. The functional analysis of these selected clones is shown in Fig. 6B. The clones which were most frequently isolated correspond to the strongest bands in the ethidium bromide-stained gel (Fig. 6A) and display the strongest dominant negative phenotype in Raji (Fig. 6b). Analysis of these clones reveals several interesting features. The clone with the smallest deletion, NLS-L102 (with the number indicating the first amino acid of CIITA retained in the clone), has lost only about half of the acid-rich region (aa 30 to 160), yet it displays a dominant negative phenotype comparable to that of NLS-D1 (aa 157). The 5′ ends of the strongest dominant negative mutants all map to a region within or just C terminal to the third P/S/T domain (NLS-L294, -L313, -L321, and -L335). The most efficient clone, NLS-L335, displays a dominant negative phenotype that is stronger than that of NLS-D5, as indicated by the comparison of the mean fluorescence intensities for HLA-DR (51.1 versus 73.1) and -DQ (13.9 versus 18). NLS-L335 probably encodes one of the shortest possible dominant negative mutants because in the ethidium bromide-stained gel the band corresponding to NLS-L335 is the smallest band detectable (Fig. 6A). Furthermore, a control mutant (NLS-L356) which starts only 20 aa downstream of NLS-L335 has no detectable dominant negative effect (Fig. 6B). Analysis of the level of protein expression shows that the NLS-L335 CIITA construct is expressed at approximately double the amount of NLS-D5 (Fig. 5, lane 4). Deletions extending beyond NLS-L335 appear to be severely affected in protein stability since we could not detect any protein corresponding to NLS-L356 or NLS-D6 by Western analysis (Fig. 5, lane 5, and data not shown).

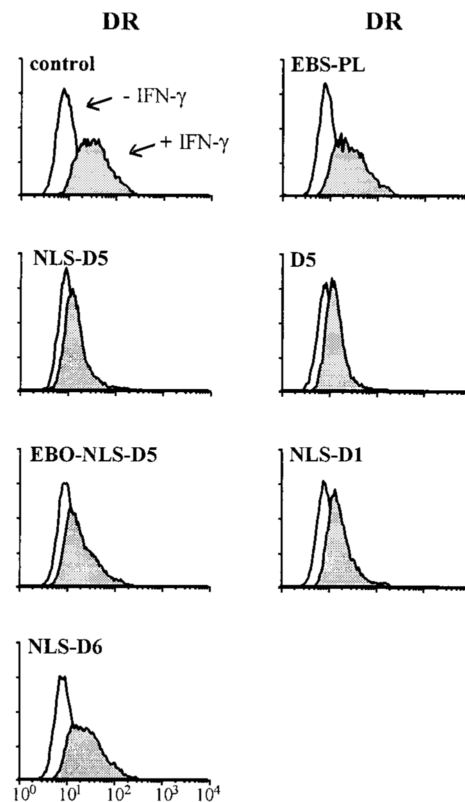


FIG. 3. Analysis of dominant negative CIITA constructs in IFN-γ induced HeLa cells. The different constructs (in EBS-PL, with the exception of EBO-NLS-D5) were transfected into HeLa cells. Stably transfected bulk cell lines were induced by IFN-γ (1,000 U/ml) and analyzed by FACS. The various constructs used are indicated. The filled profiles correspond to the IFN-γ-induced cells; the open profiles correspond to the untreated cells.

DISCUSSION

The dominant negative CIITA molecules discussed here were generated by using two different approaches. In a first step, we generated a number of CIITA mutants in a conventional manner via specific restriction sites. But because this method of generating mutants is a relatively arbitrary procedure, we set up an expression cloning strategy to functionally select dominant negative CIITA mutants from a library of random N-terminal deletions. This procedure was found to be very efficient for obtaining strong dominant negative CIITA mutants. In addition, it yielded interesting structural information. The functional selection led preferentially to the isolation of CIITA mutants with deletions of only the acidic domain (around amino acid position 160) or with truncations of both the acidic and P/S/T domains (Fig. 6A, lane 3, and 6B). This result suggests that the acidic and P/S/T-rich regions behave like two structurally distinct domains. The N termini of the strongest and most frequently isolated mutants mapped to a region at the very 3′ end of the third P/S/T-rich region (NLS-D5, -L312, -L321, and -L335 [Fig. 1 and 6]), demonstrating that both the acidic and P/S/T-rich regions are dispensable for dominant negative repression of MHC-II. Dominant negative CIITA mutants were shown by RNase protection analysis to reduce MHC-II mRNA levels very efficiently in B lymphocytes (Fig. 4). The dominant negative effect was also very strong for inducible expression in HeLa cells, where IFN-γ-induced HLA-DR expression was almost completely abolished in the case of the NLS-D5 construct (Fig. 3). We have shown by reporter gene assays that CIITA acts by activating the transcription of MHC-II genes (39). Together with the data shown here, this result indicates that dominant negative CIITA mutants function by inhibiting the transcription of MHC-II genes.

While the mode of action of CIITA remains to be elucidated, the most plausible hypothesis to date is that CIITA functions as a non-DNA binding coactivator, with its N-terminal acidic region, and potentially also the three P/S/T-rich regions, acting as a transcriptional activation domain (33, 38,

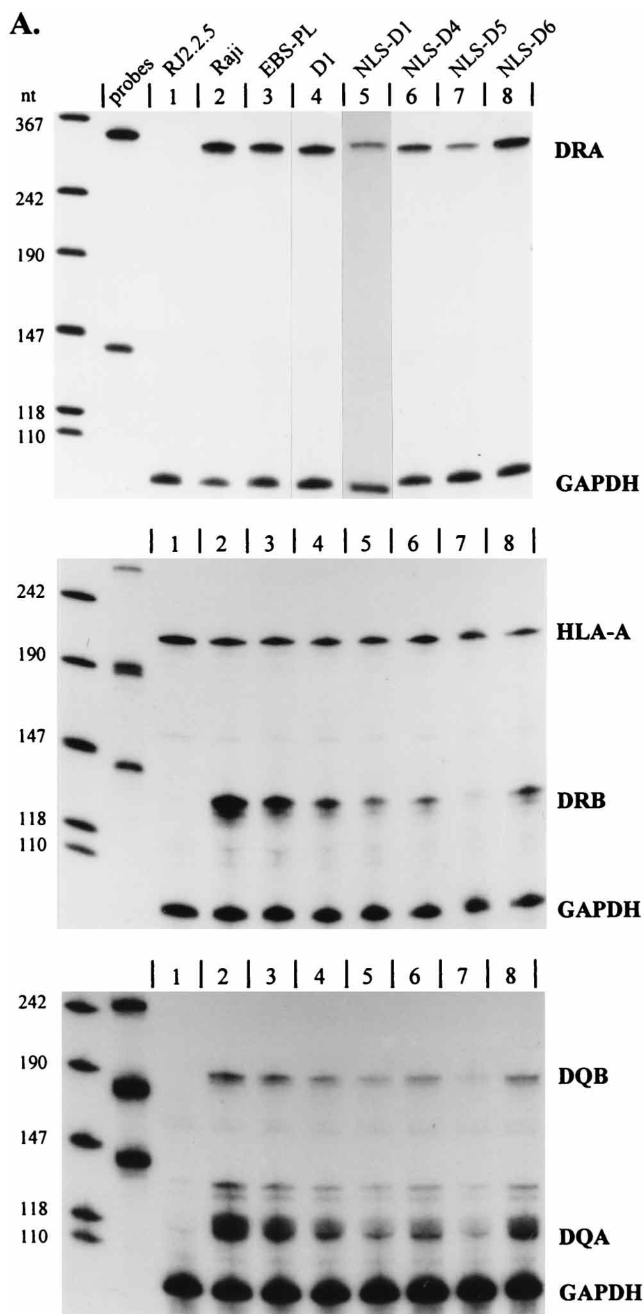


FIG. 4. Reduction of MHC-II mRNA levels through dominant negative CIITA molecules. (A) RNase protection analysis. Cytoplasmic RNAs of the various Raji transfectants indicated were analyzed by RNase protection. RNAs from the MHC-II-negative cell line RJ2.2.5 and from Raji were included as controls. Analysis of GAPDH mRNA was always included as an internal standard. The upper panel shows the analysis for HLA-DRA expression, the middle panel shows analysis for HLA-A (class I) as a control and DRB, and the lower panel shows analysis for DQB and DQA expression. The specific activity of the DRA and GAPDH probes was reduced by a factor of 5 compared to the other probes. nt, nucleotides. (B) Quantification of the results by PhosphorImager analysis. Quantification was carried out with the GAPDH signal as an internal standard. The expression levels of Raji/EBS-PL were defined as 100%. The left panel shows the expression levels of HLA-DRA, -DRB, -DQA, and -DQB mRNAs of Raji transfected with the various dominant negative CIITA constructs; analysis of HLA-A is shown on the right. NLS-D5 and NLS-D5 2nd correspond to two independent transfections.

47). Most transcription factors are comprised of several distinct functional domains, and these individual domains have often been shown to act relatively independently of each other (7). It has been shown in the case of several transcription factors that removal of the transcriptional activation domain(s) leads to a dominant negative phenotype (2, 9, 24). The fact that dominant negative CIITA molecules can be generated by removal of the acidic and P/S/T domains is in accordance with the hypothesis that CIITA acts as a transcriptional coactivator. In this context, the CIITA mutant with the smallest N-terminal deletion isolated by functional selection, NLS-L102, is rather informative. NLS-L102, in which approximately half of the acidic domain is missing, has a relatively modest but clearly detectable dominant negative effect in B lymphocytes (Fig. 6). This truncated construct is, on the other hand, also able to activate MHC-II transcription at a low level when transfected into the CIITA-deficient RJ2.2.5 cell line (data not shown). Acidic activation domains are generally considered to be largely unstructured (27), and partial truncations (for example, of the VP16 acidic activation domain) can lead to a concomitant partial reduction in transcriptional activity (34). The observation of a similar result for NLS-L102 is consistent with the idea that the acidic domain of CIITA constitutes a bona fide transcriptional activation domain.

Interestingly, when N-terminal deletions were combined with either C-terminal truncations or small internal deletions near the C terminus, the dominant negative phenotype of the mutant CIITA molecules was abolished (Fig. 1 and 2). We had established earlier that the C-terminal part of CIITA is vital for its transactivating function by showing that in two patients

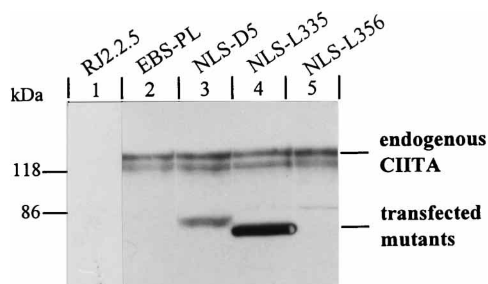
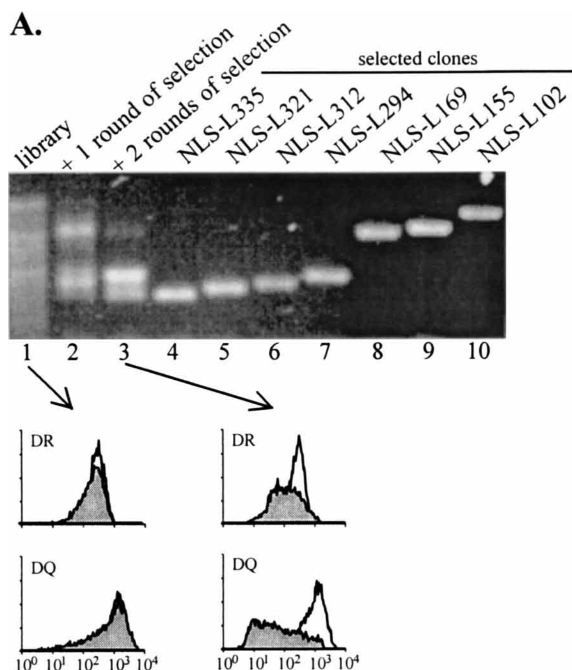


FIG. 5. Analysis of dominant negative CIITA protein expression levels by Western blotting. Total protein extracts of various stable Raji transfectants (lanes 2 to 5) and of the CIITA-deficient RJ2.2.5 cell line were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a membrane, and incubated with an anti-CIITA rabbit serum. The various dominant negative CIITA constructs, which were all transfected in EBS-PL, are indicated above the lanes. The weak band observed around 90 kDa in NLS-L356 and NLS-L335 is unspecific.



B.

selected clones

NLS-L102 NLS-L155 NLS-L169 NLS-L294 NLS-L312 NLS-L321 NLS-L335

DR

DQ

1x 2x 1x 2x 6x 5x 7x

acidic domain (30-160)

P/S/T-rich domain (162-195; 209-237; 260-322)

ATP/GTP cassette (420-427)

specific deletions

NLS-D1: aa 157 NLS-D5: aa 295 NLS-L356: aa 356

DR

DQ

10^0 10^1 10^2 10^3 10^4

FIG. 6. Isolation of dominant negative CIITA mutants by expression cloning. (A) Library selection. Gel electrophoretic analysis of the plasmid pools and of individually selected clones is shown at the top. The restriction profiles of the 5′ insert fragments of the unsorted library and after one and two rounds of selection, respectively, are shown in lanes 1 to 3. Lanes 4 to 10 show the corresponding fragments of individual plasmid clones NLS-L335, -L321, -L312, -L294, -L169, -L155, and -L102 with the numbers indicating the N-terminal amino acid of the truncated CIITA molecules. The strong band in lane 3 corresponds to an aberrant, rearranged clone (see Materials and Methods). Cell surface MHC-II expression of transfected plasmid pools before and after two transfection/sorting steps is shown in the lower part. (B) Functional analysis of individual dominant negative CIITA clones selected from the N-terminal deletion library. The N-terminal part of the predicted CIITA protein sequence is schematically represented in the middle. Positions of the acidic and P/S/T domains and the ATP/GTP binding consensus sequence are shown. The positions of the N termiini of the different constructs are indicated by vertical bars. In the upper part, the functional analysis of HLA-DR and -DQ expression by flow cytometry of the seven different plasmid species isolated from the N-terminal deletion library is shown. The relative frequencies with which the individual clones were isolated are indicated by 1x, 2x, etc. In the lower part, the phenotypes of specific deletions are shown for comparison.

with MHC-II deficiency, the disease is caused in each case by a small internal CIITA deletion in this very C-terminal region (5, 38). We show here that the dominant negative activity of CIITA mutants also depends on an intact C-terminal domain. This region of CIITA contains two so-called leucine-rich repeats which have been shown to be motifs involved in protein-protein interactions (22). On this basis, we would propose that the C-terminal region of CIITA mediates a critical contact of CIITA with protein partners, for instance, with MHC-II promoter binding proteins.

The different HLA class II isotypes (DR, DQ, and DP) are generally considered to be coregulated in a coordinated manner. However, there are a number of reports in the literature describing exceptions to this rule. In most of these cases, particularly in situations of inducible MHC-II expression, a reduced or complete absence of expression of HLA-DQ compared to HLA-DR and -DP was reported (12, 14, 18, 26). The molecular basis of this apparent discoordinated regulation is unclear. The analysis of the dominant negative CIITA mutants generated here helps to shed some light on this interesting question. Based on the cell surface analysis by flow cytometry, it appears that DQ expression is much more susceptible to the effects of dominant negative CIITA mutants than DR or DP expression. In fact, for some weak dominant negative CIITA mutants, significant effects on the cell surface could be seen only for HLA-DQ expression (Fig. 1 and 6). Astonishingly, however, this difference between HLA-DR and -DQ expression is not observed at the level of MHC-II mRNA expression (Fig. 4). Despite significant differences in overall expression levels between the different mRNAs, the relative reductions in the levels of all four MHC-II mRNA species analyzed were very similar (Fig. 4). This was true both for dominant negative CIITA mutants with a weak phenotype and for those causing strong repression. The mRNA analysis thus reveals that CIITA is of equal importance for both isotypes DR and DQ qualitatively as well as quantitatively, and we see no evidence for a discoordinated, isotype-specific transcriptional regulation by CIITA. It follows from these observations that the apparent discrepancy between the mRNA and cell surface expression levels are most probably due to a posttranscriptional mechanism acting differentially on DR and DQ. Such differential posttranscriptional effects have also been observed in interspecies hybrids between RJ2.2.5 and murine B cells (11). The results obtained here by a completely different approach lead to a similar conclusion, suggesting that these posttranscriptional differences between DR and DQ are a biologically relevant phenomenon.

The mutants discussed here, in which part of the protein has been deleted, most probably constitute loss-of-function mutants executing their dominant negative function through the sequestration of protein partners. This mechanism generally necessitates overexpression of the dominant negative mutant with respect to the wild-type protein. Only under particular circumstances can it be expected that dominant negative mutants will be effective when present in stoichiometric concentrations. One of these possibilities is that the protein in question acts as a homomultimer and that functional integrity of all subunits is necessary for function (19). An example of this kind is the tumor suppressor p53, which acts as a homotetramer and is very efficiently blocked by dominant negative loss-of-function mutants (6, 17, 43). Another possibility is a dominant negative mutant in which a loss-of-function mutation is combined with a gain-of-function mutation leading, for example, to increased stability of a protein-protein interaction. It was therefore not unexpected to observe that the most efficient dominant negative CIITA mutants discussed here are considerably overexpressed with respect to the wild-type protein (Fig. 5). This observation can also suggest that CIITA does not act as a homomultimer.

Comparison of NLS-L335 and NLS-D5 shows that dominant negative strength is correlated with the level of protein expression (Fig. 5 and 6). It is also noteworthy that the N termini of two of the strongest library-selected mutants (NLS-L312 and NLS-L335) precisely overlap with exon boundaries (5, 8, 42). Exon boundaries are known to delimit frequently structural domains. It is therefore possible that precise truncation of the mutant CIITA proteins at exon boundaries facilitates protein folding and leads to increased protein stability. Relatively subtle differences in the N-terminal ends of the mutant proteins may favor or inhibit proper protein folding and may therefore have relatively drastic effects on overall protein levels. This is in accordance with our finding that mutants with deletions starting only a few amino acids downstream of NLS-L335 were undetectable at the protein level (Fig. 5 and data not shown).

The functional selection strategy developed here for the generation of dominant negative CIITA mutants was found to be very efficient. The isolation of strong dominant negative mutants from a complex mixture of plasmids was straightforward, and due to the dilution strategy used, the number of false positive clones isolated was negligible. The efficiency of the procedure shown here raises hopes that it could be used to isolate novel gain-of-function mutants of CIITA with even stronger dominant negative phenotypes from randomly mutated CIITA libraries. These should be very useful for functional experiments and should also provide valuable structural information. For example, increased interaction with critical protein partners would not only identify crucial regions of CIITA but also facilitate the isolation of such unknown partners.

Finally, the strongest dominant negative CIITA mutants isolated here, particularly NLS-L335, are probably strong enough to lead to significant reductions in T-cell stimulation when used in in vivo experiments. The efficiency of T-cell stimulation has been shown to depend quantitatively on the amounts of stimulatory MHC molecules that are expressed (20). Experimental inhibition of MHC-II expression should be informative in, for example, animal models of autoimmune diseases, in which MHC-II-dependent CD4 T-cell-mediated immune responses have been shown to play a critical role in pathology (1, 21). Since the role of aberrant MHC-II expression in the target tissues of autoimmune reactions is still very much the subject of controversy, it may be very informative to reduce this inducible expression in a tissue-specific manner through dominant negative CIITA introduced into transgenic mice. Another promising field of application for dominant negative CIITA mutants may be xenotransplantation. Especially for free tissue grafts such as pancreatic islets or skin, containment of the cellular xenoimmune response against the donor organ may be of importance for graft survival (30). It has been shown that porcine tissue can elicit a strong human anti-porcine xeno-MHC response, which is directed mostly toward porcine MHC-II antigens (46). Since gene inactivation through homologous recombination is currently not available for pigs, transgenic dominant negative CIITA mutants may be a convenient way to reduce MHC-II expression, and thus immunogenicity, in porcine donor tissue.

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