Nuclear Appearance of a Factor That Binds the CD28 Response Element within the Interleukin-2 Enhancer Correlates with Interleukin-2 Production*

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Azem Civilt, Arjen Bakker§, Irma Rensink¶, Stefan Doerre¶, Lucien A. Aardent, and Cornelis L. Verweij¶

From the §Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Department of Autoimmune Diseases, Amsterdam, 1066 CX The Netherlands, the ¶Department of Microbiology, Boston University, Boston, Massachusetts 02118-2394, and the ¶Department of Rheumatology, Academic Hospital Leiden, Leiden, 2300 RC The Netherlands.

Activation of T lymphocytes requires the combined signaling of the T cell receptor and costimulatory molecules such as CD28. The ability of T cells to produce interleukin-2 (IL-2) is a critical control point in T lymphocyte activation. The IL-2 enhancer contains a functional motif named CD28 response element (CD28RE) that serves as a target for mitogenic T cell activation signals. The CD28RE sequence reveals similarity to the consensus xB binding motif. Here we demonstrate that CD28RE binds an inducible protein with a molecular mass of approximately 35 kDa called nuclear factor of mitogenic-activated T cells (NF-MATp35) that is clearly different from the known NF-xB/Rel family members. Induction of NF-MATp35 was shown to depend on de novo protein synthesis and was restricted to T cells that received a mitogenic combination of T cell stimuli, not necessarily including CD28 signaling. Nonmitogenic T cell stimulation did not result in appearance of NF-MATp35. These results indicate that mitogenic combinations of T cell activation signals are integrated at the level of NF-MATp35 induction. Similar to its effect on IL-2 production, cyclosporin A inhibited the induction of NF-MATp35. Taken together, these data demonstrate that the nuclear appearance of NF-MATp35 shows excellent correlation with IL-2 production, which is a unique characteristic among nuclear factors implicated in the control of IL-2 gene expression.

Proliferation and maturation of resting T lymphocytes is an essential process in the T cell-mediated immune response. Resting T lymphocytes can be activated via noncovalent interactions of the T cell antigen receptor (TCR) complex with a peptide in association with major histocompatibility complex molecules. Besides TCR signaling, a second antigen-nonspecific costimulatory is required for optimal T cell activation, which is accompanied by the production of high levels of interleukin-2 (IL-2) (1–3). IL-2 serves a function as autocrine growth factor for T cells allowing them to enter the S phase (4, 5). Occupancy of the TCR without a costimulatory signal leads to abortive T cell activation and the development of functional unresponsiveness or clonal anergy of T cells in which the T cells are incapable of producing IL-2 (1, 6). Therefore, the ability of T cells to synthesize IL-2 is a critical control point in determining their participation in an immune response and consequently serves as a model system for the analysis of molecular events in T cell activation (7).

Considerable evidence has indicated that signals delivered through the T cell accessory molecule CD28 constitute a major costimulatory pathway (8). This costimulatory signal is induced upon interaction of CD28 with its counterreceptors CD80 and CD86 that are expressed on the APC (9, 10). Monoclonal antibodies directed against CD28 have been shown to serve as a valid substitute to mimic the CD80-CD86 interaction in CD28 triggering (11, 12). Monoclonal anti-CD28 antibodies either cooperate with soluble anti-CD3, which simulates antigen-specific TCR triggering, or synergize with protein kinase C for the induction of IL-2 production and T cell proliferation. Unlike the TCR-induced signaling pathway, the CD28 signal transduction route does not involve the formation of inositol 1,4,5-trisphosphate or translocation of protein kinase C and is resistant to the immunosuppressive effects of cyclosporin A (CsA) (8, 13). Recent evidence suggests that the CD28-induced signal is mediated via phosphatidylinositol 3-kinase (14–16). In addition, Su and colleagues (17) reported that simultaneous activation of TCR and CD28 results in a synergistic activation of c-Jun kinase. The latter observation implies that TCR and costimulatory signals become integrated at the level of c-Jun kinase activation.

Human T cells respond to CD28 costimulation by a dramatic enhancement in the induction of IL-2 mRNA. Two mechanisms account for the CD28 co-induced expression of the IL-2 gene. CD28 costimulation has been shown to activate IL-2 gene transcription (18, 19) and to prolong IL-2 mRNA half-life (20, 21). Regulation of IL-2 gene transcription is controlled by an enhancer extending from –52 to –319 relative to the transcription initiation site (22, 23). Specific regulatory sequences that bind the nuclear factors AP-1, NF-xB, Oct-1, and NF-AT1 have been implicated in regulation of IL-2 gene transcription (reviewed in Ref. 24). We and others have identified a sequence from –162 to –153 (5’-AGAAATTCACA-3’) within the IL-2 enhancer that serves a crucial role as a response element for mitogenic combinations T cell activation signals (18, 19, 25) (Fig. 1). The sequence of the CD28RE motif is strongly conserved between the human and murine IL-2 gene. Comparison...
Identification of NF-MAT

of the sequence with that of binding motifs of known transcription factors reveals similarity to the consensus NF-κB binding motif (26, 27). Only the first and the last (tenth) position differ from the consensus NF-κB binding motif (19). The significance of the sequence similarity has been shown by cross competition studies using CD28RE and the HIV-1 κB motif in combination with nuclear extracts of mitogen-activated cells. The HIV-1 κB motif efficiently competed for CD28RE binding activity, whereas CD28RE proved about 25-fold less effective in competition for HIV-1 κB binding activity than the HIV-1 κB motif itself. These data suggest that proteins involved in interacting with CD28RE are also involved in binding the HIV-1 κB motif.

In vitro binding studies have demonstrated that induction of CD28RE binding activity is restricted to T cells that received a mitogenic combination of T cell activation signals, not necessarily including CD28 (25, 28). In order to characterize the proteins that constitute the CD28RE binding activity, we performed UV cross-linking analysis. In this manuscript we describe the characterization of a protein called NF-MATp35 (nuclear factor of mitogenic activated T cells) that binds CD28RE. Although evidence is presented that NF-MATp35 also binds to the κB motif, NF-MATp35 is clearly distinct from the known NF-κB/Rel family members. It is demonstrated that the induction of NF-MATp35 shows excellent correlation with IL-2 production.

MATERIALS AND METHODS

Cell Culture—The human T cell leukemia line Jurkat was cultured in Iscove’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were stimulated in Iscove’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin. Cells were stimulated with nuclear extracts of mitogenic-activated cells. The HIV-1 κB motif efficiently competed for CD28RE binding activity, whereas CD28RE proved about 25-fold less effective in competition for HIV-1 κB binding activity than the HIV-1 κB motif itself. These data suggest that proteins involved in interacting with CD28RE are also involved in binding the HIV-1 κB motif.

FIG. 1. A, schematic representation of the IL-2 enhancer with the known protein binding sites (24, 45, 46). The location of these sites is given by numbers that represent the position in base pairs relative to the site of initiation of transcription. B, comparison of the human and murine IL-2 CD28RE sequence. C, sequence of CD28RE is compared with the consensus NF-κB binding sequence.
CD28RE-3, 5'-gatcAGAAATT*CCAAgatc-3' and 3'-ctagTTCT*T-TAAGTTTTctag 5'; HIV-1 κB, 5'-gatcGAGGGGACT*TTCCgatc-3' and 3'-ctagCTCCCC*GAAAGGctag 5'.

DNA binding reactions were performed by incubation of 12 μg of nuclear extract with the BrdUrd-substituted probe (1-5 x 10^7 cpm) in the presence of poly(dI-dC) as a nonspecific competitor. Nucleoproteins were first resolved by gel retardation prior to UV irradiation in situ (UV Stratalinker, Stratagene). Covalently linked nucleoprotein complexes were eluted from the gel and further analyzed by 10% SDS-polyacrylamide gel electrophoresis after precipitation with acetone. For immunoprecipitation the eluted proteins were mixed with 1 μl of undiluted antiserum and protein A-Sepharose beads. The bound proteins were washed with radiomimune precipitation buffer and analyzed by SDS-polyacrylamide gel electrophoresis (44).

RESULTS

Characterization of CD28RE-binding Proteins by UV Cross-linking—Using gel retardation analysis, we and others (18, 19, 25, 28) have demonstrated specific binding of an inducible nuclear factor with the CD28RE motif. The CD28RE binding factor was found to be selectively induced by distinct mitogenic combinations of T cell stimuli, not necessarily including CD28 signaling (25, 28) (Fig. 2A). We named this factor NF-MAT, which stands for nuclear factor of mitogenic activated T cells.

To characterize the inducible nucleoprotein(s) that constitutes NF-MAT, we performed UV cross-linking experiments with a 5’-bromo-2’-deoxyuridine-substituted CD28RE probe. Nuclear extract of Jurkat cells stimulated with anti-CD3 plus anti-CD28 was incubated with this probe, and the protein-bound DNA fraction was separated by gel retardation and subjected to in situ UV irradiation. Analysis of the cross-linked protein-DNA complexes by SDS-polyacrylamide gel electrophoresis revealed the presence of two protein-DNA bands that migrated with molecular masses of 35 and 70 kDa, respectively (Fig. 2B).

Next we applied UV cross-linking analysis to confirm that the CD28RE-protein complex that appeared upon stimulation of Jurkat cells via different modes of mitogenic T cell activation revealed the same pattern of protein-DNA bands as we observed for the complex induced by the combination of anti-CD3 and anti-CD28 (Fig. 2B). Because the CD28RE-binding proteins selectively showed up in nuclei of mitogenically activated T cells, we refer to the proteins that correspond to the 35- and 70-kDa cross-linked adducts as NF-MATp35 and NF-MATp70, respectively. The actual molecular mass of these proteins could be slightly lower than that of the respective protein-DNA complexes due to the absence of covalently bound oligonucleotide.

NF-MATp35 May Bind CD28RE as a Dimer—Many nuclear proteins have been shown to bind their cognate binding motif as a dimer (26, 27, 29). Both homo- and heterooligomers have been shown to be involved in DNA binding. To explore the possibility that NF-MATp35 represents the dimeric form of NF-MATp35, we performed UV dose titration experiments (Fig. 3). These experiments revealed that upon short exposure (i.e., 1 min) the 35-kDa band predominated, whereas the balance shifted toward the 70-kDa band during long exposure (15 min). This result suggests that the protein present in the 70-kDa complex represents the homodimer of the 35-kDa protein moiety.

NF-MATp35 Is Distinct from the Known NF-κB/Rel Family Members—The CD28RE sequence revealed similarity to the NF-κB consensus sequence (18, 26, 27). Based on the ability of κB motifs to compete for NF-MAT binding, we determined whether NF-κB immunoreactivity could be detected in the UV cross-linked CD28RE-protein complex. In Fig. 4A it is demonstrated that none of the antibodies directed against the individual NF-κB/Rel proteins NF-κB1, Rel-A, and c-Rel were able to immunoprecipitate CD28RE cross-linked protein. The capacity of these antibodies to immunoprecipitate NF-κB/Rel-DNA complexes was demonstrated using recombinant NF-κB/Rel proteins in combination with a BrdUrd-incorporated HIV-1 κB probe (Fig. 4B).

Evidence for Binding of NF-MATp35 to the HIV-1 NF-κB Motif—NF-κB/Rel proteins are known to be efficiently expressed in T cells receiving a single stimulus. In analogy with
these observations, anti-CD3 or PMA alone proved to be effective in the induction of HIV-1 \( \kappa B \) motif binding activity (Fig. 5A). When HIV-1 \( \kappa B \) protein complexes formed in nuclear extracts of cells that received a single stimulus were compared with those from cells that were treated with a mitogenic combination of stimuli, we observed both quantitative and qualitative differences. Complexes that appeared in nuclear extracts of cells that were treated with a mitogenic combination of stimuli revealed increased intensity and were extended with a form exhibiting enhanced mobility (C2).

In order to determine whether NF-MATp35 contributes to the HIV-1 NF-\( \kappa B \)-protein complex, we performed UV cross-linking experiments with a BrdUrd-incorporated HIV-1 \( \kappa B \) motif. With nuclear extracts of nonmitogenic activated T cells, two major protein-DNA bands with molecular masses of approximately 70–85 kDa and 50 kDa, respectively, were observed (Fig. 5B). HIV-1 \( \kappa B \)-protein complexes that were formed in nuclear extracts of cells that have received a mitogenic combination of activation signals contained an additional protein-DNA product with a molecular mass identical to that of the NF-MATp35-CD28RE complex. Distinct mitogenic combinations of T cell activation conditions led to the inclusion of this protein in the HIV-1 \( \kappa B \)-protein complex. Similar to the NF-MATp35-CD28RE complex, the 35-kDa protein-HIV-1 \( \kappa B \) complex could not be immunoprecipitated with anti-NF-\( \kappa B \)1, anti-RelA, and anti-c-Rel antibodies (data not shown).

Induction of NF-MATp35 Is Dependent on de Novo Protein Synthesis—To determine whether nuclear appearance of NF-MATp35 is dependent on de novo protein synthesis, we investigated the effects of the protein synthesis inhibitor cycloheximide on the mitogen-inducible nuclear appearance of NF-MATp35. The addition of cycloheximide 30 min prior to stimulation with anti-CD3 and anti-CD28 led to a dramatic reduction in the appearance of NF-MATp35 (Fig. 6). This result indicates that either NF-MATp35 itself must be synthesized or that another protein needs to be synthesized that activates a pre-existing but inactive form of NF-MATp35.

When the HIV-1 \( \kappa B \) motif was used as a probe, cycloheximide selectively inhibited the appearance of the fast migrating moiety (C2) of the mitogenic induced complex (Fig. 7A). The addition of anti-serum directed against NF-\( \kappa B \)1 reveals that the complex that appears with extracts of nuclei from mitogenically stimulated (anti-CD3 plus anti-CD28) cells in the presence of
cycloheximide resembles the complex induced upon nonmitogenic stimulation with anti-CD3 alone (Fig. 7B).

UV cross-linking analysis with a BrdUrd-substituted HIV-1 xB motif shows that the complex that appears in the presence of cycloheximide is devoid of the p35 protein (Fig. 6B). This result is in accordance with the NF-MAT data and substantiates the conclusion that the protein that constitutes the 35-kDa protein-HIV-1 xB complex is identical to NF-MATp35.

**DISCUSSION**

In this study we describe an approximately 35-kDa inducible protein, named NF-MATp35, which interacts with the CD28RE within the IL-2 enhancer. Distinct mitogenic combinations of T cell activation signals contribute to the nuclear appearance of NF-MATp35, indicating that there is a redundancy in the activation requirements of NF-MATp35.

We previously reported that the CD28RE sequence displays similarity with the xB enhancer consensus motif. The Rel-related proteins c-Rel, NFkB1, and RelA constitute a major class of xB enhancer-binding proteins. In line with this Ghosh...
etal. (28) were able to detect the presence of NFκB1, RelA, and c-Rel among the proteins that bind CD28RE. However, the affinity for NFκB/Rel proteins is low as judged from competition experiments and UV cross-linking studies performed by us and others (19, 30, 31).

Our data clearly demonstrate that NF-MATp35 is distinct from NFκB1, RelA, and c-Rel. First, the activation requirements of NF-MATp35 are more stringent than those for the induction of the NFκB/Rel family members. Second, the molecular mass of NF-MATp35 does not match those of the individual NFκB/Rel proteins. Third, antibodies directed against NFκB1, RelA, or c-Rel have not been successful in immunoprecipitating NF-MATp35 covalently linked to CD28RE. Finally, the appearance of NF-MATp35 is dependent on protein synthesis. It is interesting that Fraser and Weiss (30) reported that the predominant protein species present in the CD28RC constitute three proteins of 35, 36, and 44 kDa. The most abundant were the 35/36-kDa proteins. It could be that NF-MATp35 is related to one of these proteins; however, in contrast to NF-MATp35, the CD28RC proteins require the contribution of the CD28 signaling for their nuclear appearance. In addition, none of these proteins appears upon stimulation with anti-CD28 plus PMA but requires the addition of a third stimulus, i.e. ionomycin. Furthermore, Li and Siekevitz (31) described a 45-kDa Tax inducible protein (TxREF) distinct from NFκB/Rel that specifically interacts with CD28RE. This protein could be related to the 44-kDa protein described by Fraser and Weiss (30).

The low affinity of CD28RE for binding NFκB/Rel proteins could be explained by differences in the 5' and 3' extremities between CD28RE and NFκB/Rel binding motifs. Analysis of the various NFκB/Rel binding motifs has showed that the 5' and 3' ends of the recognition motif are the best conserved parts (32). Based on crystallographic analysis of NFκB-DNA complexes, it is believed that these parts of the κB binding motif in particular are involved in NFκB/Rel binding (33, 34). The fact that NF-MATp35 binds to the κB elements as well as to CD28RE, whereas NFκB/Rel proteins have a preference for the consensus-matching sequences, indicates that less variation is tolerated for binding of NFκB/Rel proteins than for NF-MATp35. This might implicate that every κB element is in potency able to bind NF-MATp35.

A functional role of CD28RE as a mitogen response element in the activation of the IL-2 enhancer stems from studies wherein a mutant enhancer with a nonfunctional CD28RE site

![Fig. 7. Influence of cycloheximide and CsA on the appearance of mitogenic induced HIV-1κB binding activity. A, gel retardation analysis was performed with HIV-1κB as a probe, and nuclear extract from Jurkat cells was stimulated for 8 h in the absence (lanes 1) or the presence of either cycloheximide (lanes 2-4) or CsA (lanes 5-7). Lane 1, cells stimulated with anti-CD3 plus PMA; lanes 2 and 5, anti-CD3 plus PMA; lanes 3 and 6, anti-CD3 plus PMA; lanes 4 and 7, anti-CD28 plus PMA. B, gel retardation assay was performed with HIV-1κB as a probe in the absence (lanes 1-4) or the presence (lanes 5-8) of antibodies directed against NFκB1 with nuclear extract from stimulated Jurkat cells. Lanes 1 and 5, cells stimulated for 8 h with anti-CD3; lanes 2 and 6, anti-CD3 plus anti-CD28; lanes 3 and 7, anti-CD3 in the presence of cycloheximide; lanes 4 and 8, anti-CD3 plus anti-CD28 in the presence of cycloheximide.](http://www.jbc.org/)

| Table I |
| Summary of requirements for induction of IL-2 production and nuclear expression of NF-MAT |
| --- |
| **Stimulus** | **IL-2** | **NF-MAT** |
| αCD3/αCD28 | + | + |
| αCD3/αCD28/CsA | - | - |
| αCD28/PMA | + | + |
| αCD28/PMA/CsA | + | + |
| αCD3/PMA | + | + |
| αCD3/PMA/CsA | - | - |
| PMA | - | - |
| αCD3 | - | - |
| αCD28 | - | - |
was used in transient expression studies (18, 19). In accordance with the requirements for NF-MATp35 induction, mutations within the CD28RE motif affected the IL-2 enhancer in its capacity to respond to mitogenic T cell activation signals. Because CD28RE serves as a response element for distinct mitogenic combinations of T cell activation signals not necessarily including CD28 signaling, we propose to name the element MARS, which stands for mitogenic activation responsive sequence (35).

IL-2 production is either completely resistant (anti-CD28 plus PMA) or sensitive (anti-CD3 plus either anti-CD28 or PMA) to the immunosuppressive action of CsA (13, 36, 37). Inhibition of IL-2 gene expression by CsA is explained by the negative effects of CsA on the nuclear expression of the cytoplasmic component of NF-AT and the activation of Oct-1/AP1, both known to be TCR-responsive nuclear proteins (37–40). Here we demonstrated that nuclear appearance of NF-MATp35 is also sensitive to CsA. The sensitivity of NF-MATp35 for CsA is, similar to IL-2 gene expression, dependent on the mode of mitogenic stimulation. When Ca²⁺ influx is not involved in the signaling regime, NF-MAT still appears. Taken together the above mentioned results show that the induction of NF-MATp35 reveals excellent correlation with activation of IL-2 gene expression (Table I). This is a unique characteristic among nuclear proteins implicated in IL-2 gene regulation and suggests a crucial role for NF-MATp35 in IL-2 gene regulation.

Similar to the induction of NF-MATp35, the activation of c-jun kinase revealed excellent correlation with IL-2 production (17). However, based on supershift analysis and immunoprecipitation experiments with anti-c-jun antibodies, we could exclude the possibility that NF-MATp35 is identical to c-jun.2 c-jun kinase is the only protein kinase among the kinases involved in mitogenic signaling described so far that requires mitogenic combinations of T cell stimuli for its activation. This indicates that signal integration occurs at an even more proximal step in the events that lead to IL-2 gene transcription than NF-MATp35 induction. Therefore, it is of interest to investigate whether NF-MATp35 is somehow involved in c-jun kinase signaling.

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