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Regulation of Transcriptional Activity of the Murine CD40 Ligand Promoter in Response to Signals Through TCR and the Costimulatory Molecules CD28 and CD2

Hanna Lindgren, Karol Axcrona, and Tomas Leanderson

We have analyzed the murine CD40 ligand promoter with regard to stimulation of transcriptional activity in Jurkat T cells after signaling via the TCR and the costimulatory molecules CD28 and CD2. TCR engagement was necessary for the induction of transcriptional activity from the CD40 ligand promoter, and costimulation through either CD28 or CD2 further increased the activity. Analysis of promoter deletants showed that the DNA elements needed for transcriptional activity induced by costimulatory molecules were located within two regions containing previously identified transcription factor NFAT sites. Further studies of the proximal NFAT site showed that it was not dependent on AP-1 binding for transcriptional activity induced by costimulation through CD28. Instead, a region between the TATA box and the proximal NFAT site was shown to bind proteins of the early growth response family and to contribute to NFAT-mediated transcriptional activation. The Journal of Immunology, 2001, 166: 4578–4585.

During T lymphocyte activation by APCs, cross-talk between surface bound receptor-ligand pairs as well as soluble mediators is required. The primary signal is mediated through MHC binding to the TCR, and examples of costimulatory signals are those delivered through the surface molecules CD28 and CD2 by ligation to B7 and LFA-3 present on the APC surface (1, 2). Another costimulatory molecule, needed for activation of APCs, is the CD40 ligand (CD40L) that is transiently expressed on the surface of activated Th cells (for review, see Refs. 3 and 4). The CD40L interacts with CD40 that is expressed on B cells (5), macrophages (6), and dendritic cells (7, 8). A CD40 signal is required for germinal center development (9, 10) and will rescue germinal center B cells from apoptosis and promote B cell differentiation (9, 11, 12). On dendritic cells, ligation of CD40L to CD40 results in up-regulation of costimulatory molecules and enhancement of cytokine production (13–16). CD40L expression on the T cell surface has been shown to be induced by TCR interactions with MHC class II on the APC surface and further stabilized by costimulation through B7/CD28 interactions (12, 17–19). Signaling through CD40L also has distinct effects on T cell activation and may regulate the T cell response qualitatively (20–24).

Both costimulation through CD28 and CD2 induce nuclear translocation of the transcription factor NFAT (25, 26). NFAT has been shown to interact with sites in promoters controlling expression of several genes that are important during T cell activation, including IL-2 (27), IL-4 (28), and CD40L (29, 30). NFAT was initially described as a cyclosporin A-sensitive factor (31, 32), and four different forms of NFAT have been identified that share conserved DNA binding domains and require similar activation signals (for review, see Ref. 33). Recently, a fifth member of the NFAT family (NFAT5) was cloned that is constitutively localized in the nucleus and differs in structure, DNA binding, and regulation from the other NFAT family members (34). Transcription factors of the Jun and Fos families, together forming the transcription factor AP-1, have been demonstrated to interact with both NFAT and octamer-binding protein (Oct)-1 in the IL-2 promoter (35–38) and with NFAT in the CD40L promoter (30).

In this paper we show that induction of the CD40L and IL-2 promoters differs in response to costimulatory signals despite the similarities in transcription factor binding sites between the two promoters. We show that the CD40L promoter is induced both by TCR ligation alone and by additional signaling through CD28 and CD2, while the IL-2 promoter is only induced by the combination of signals through TCR and costimulatory molecules. Furthermore, the induction of CD40L promoter activity is dependent on two NFAT sites in the promoter, whereas the proximal site is not dependent upon interactions with AP-1 to be transcriptionally active. Instead, a region located 3′ of the TATA-proximal NFAT site is important for transcriptional activity induced by signals through TCR and CD28, and we identify proteins of the early growth response (Egr) family of transcription factors to bind to this region.

Materials and Methods

Reagents

Staphylococcal enterotoxin E (SEE) was purchased from Toxin Technology (Sarasota, FL). PMA and ionomycin were purchased from Sigma (Sigma-Aldrich, St. Louis, MO). mAbs directed to mouse NFATp and NFATc were purchased from BD PharMingen (San Diego, CA). The polyclonal Abs against p50, p65, Egr-1, Egr-2, and Egr-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell lines and cell culture

Chinese hamster ovary (CHO) cells transfected with cDNA for HLA-DR4, B7-1, and LFA-3 were used as previously described (39, 40). The different CHO cells used in this study were CHO-DR (single transfected to express the HLA-DR molecule on the surface), CHO-DR/B7-1, or CHO-DR/B7-1, and LFA-3 were used as previously described (39, 40). The different
LFA-3 (double transfected to express both HLA-DR and human B7-1 or LFA-3) and CHO-DR/CHO-B7-1/LFA-3 (triple transfected to express HLA-DR, human B7-1, and human LFA-3). Single, double, and triple transfec- 
ts expressing similar levels of the transfected molecules were obtained by repeated cell sorting. The CHO cells were maintained in culture in RPMI 1640 medium without glutamine supplemented with 10% FCS, 20 mM HEPES, 2 mM sodium pyruvate, 50 μM 2-ME, and 50 μg gentamicin/ml (all from Life Technologies, Taby, Sweden). The human Jurkat leukemia T cell line was maintained in culture in RPMI 1640 medium supplemented with 1% FCS, 2% M HEPS, 2 mM sodium pyruvate, 50 μM 2-ME, and 50 μg gentamicin/ml (all from Life Technologies). All tests on the Jurkat T cells were performed at a concentration of 10^6 cells/ml. The CHO transfectants were added to the Jurkat cells at a concentration of 10^5 cells/ml. All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2.

**Reporter gene constructs**

An ~1600-bp fragment of the murine CD40L promoter was excised from a mouse genomic clone of the CD40L gene with XhoI/BglII and subcloned into pGem3Z (Promega, Madison, WI). The full-length promoter was generated by PCR amplification from the pGem3Z plasmid containing the CD40L promoter using the Sp6 primer. The amplified product was cloned into a BglII/HindIII-opened pGL-2 Basic promoter and enhancerless luciferase reporter plasmid (Promega). The resulting construct was named pGL-X. CD40L promoter deletions were PCR amplified from pGL-X with a 3’ reverse primer containing a HindIII site just 5’ of the start codon ATG-5’-GAGAAGCTTCGCTGACTGAGAACCTGAAA-3’. Corresponding 5’ primers containing a BglII site linked to the 5’ end were used. The amplified products were cloned into a BglII/HindIII-opened pGL-2 Basic vector. The 5’ primers used for generating the deletants were: 5’-GAGAGATCTGAGGAGCAGGTGGTATTATCTTTCTC-3’ (pGL-I), 5’-GAGAGATCTTTCCTTGTATACCCAGTCCAGG-3’ (pGL-II), 5’-GAGAGATCTGAGGAGAAGCGTCCGCTCCG-3’ (pGL-III), 5’-GAGAGATCTGAGGAGAAGCGTCCGCTCCG-3’ (pGL-IV), 5’-GAGAGATCTTTCCTTGCTTACCGACCAGAAGGCG-3’ (pGL-V), 5’-GAGAGATCTTTCCTTGCTTACCGACCAGAAGGCG-3’ (pGL-NFAT), 5’-GAGAGATCTTTCCTTGCTTACCGACCAGAAGGCG-3’ (pGL-NFAT), 5’-GAGAGATCTTTCCTTGCTTACCGACCAGAAGGCG-3’ (pGL-NFAT), and 5’-GAGAGATCTTTCCTTGCTTACCGACCAGAAGGCG-3’ (pGL-NFAT).

**Transient transfections and luciferase activity analysis**

Transfection of Jurkat T cells was conducted using the lipofectin method as described by the manufacturer (Life Technologies). Briefly, 2 μg of plasmid DNA was mixed with 10 μl of lipofectin in 400 μl of OptiMEM medium and added to 3 x 10^5 Jurkat cells. The transfected cells were incubated for 22 h, and the cells transfected with the same promoter deletant were pooled. The Jurkat cells (1 x 10^6 cells/ml) were stimulated in triplicate with the different CHO transfected cells (1 x 10^5 cells/ml) in the presence or the absence of 100 ng of SEE/ml, or they were left unstimulated. After 8 h of incubation the cells were harvested, washed twice in PBS, and treated with 100 μl of reporter lysis buffer according to the manufacturer’s recommendations (Promega). Twenty microliters of each lysate was assayed for luminescence with luciferase assay substrate (Promega) and counted in a Wallac 1250 Microbeta counter (Wallac, Turku, Finland). The resulting luminescence was corrected for protein content determined by BCA assay (Pierce, Rockford, IL). All experiments were performed in triplicate, and the mean value ± standard error of the mean (SEM) is presented.

**EMSA and nuclear extracts**

Jurkat cells (1 x 10^6 cells/ml) were stimulated with the different CHO transfected cells (1 x 10^5 cells/ml) in the presence of 100 ng of SEE/ml or with PMA (50 ng/ml) and ionomycin (1 μM) for 6 h, or they were left unstimulated. Nuclear extracts were prepared according to the method reported by Schreiber et al. (43). Oligonucleotides were labeled with [γ-32P]ATP by incubation with T4 polynucleotide kinase (Roche Diagnostics Scandinavia, Bromma, Sweden), annealed to the antisense strand, and purified on 5% polyacrylamide Tris-borate-EDTA gel. Nuclear extracts were incubated with 2 μg of polyclonal anti-TNF receptor antibody for 15 min at room temperature in binding buffer (for the NFAT, CD40L 3’ deletant, and NFAT/Egr oligonucleotides: 20 mM HEPES (pH 7.9), 40 mM KCl, 2.5 mM MgCl2, 1 mM DTT, and 4% Ficoll; for the Oct oligonucleotide: 10 mM HEPES (pH 7.9), 70 mM KCl, 1 mM DTT, 1 mM EDTA, and 2.5 mM MgCl2). Probe (20,000 cpm) was added to each sample, and the incubation was continued for 30 min at room temperature. For cross-competition experiments unlabeled oligonucleotides were added 10 min before addition of radio labeled oligonucleotide. For supershifts the mixture of nuclear extracts and radio labeled oligonucleotide was incubated with 0.5 μg of NFATp or NFATc Abs; 4 μg of Egr-1, Egr-2, or Egr-3 Abs; or 2 μg of the control Abs (p50 or p65) for 1 h on ice. The samples were separated on a 5% polyacrylamide Tris-borate-EDTA gel, which was subsequently dried and analyzed by autoradiography. Oligonucleotides used for EMASAs were as follows: CD40L NFAT (proximal site of the murine promoter), 5’-AAGGACACACCTTGAGGAGAAGCTTCGCTGACTGAGAACCTGAAA-3’, CD40L NFAT extended, 5’-GAAGCTTACAGAACAGTTCGCTGACTGAGAACCTGAAA-3’.

**Results**

Activity of the CD40L promoter is induced by TCR ligation and enhanced by costimulation through CD28 or CD27, while induction of IL-2 promoter activity requires costimulation through TCR and CD28.

The objective of this study was to define the sequence elements and signal requirements for efficient transcriptional activation of CD40L expression using a transient transfection approach. We used the Jurkat T cell line as a target for our transfections and stimulated the transfecants with various combinations of MHC II and costimulatory molecules using CHO cells transfected with HLA-DR, HLA-DR/DR7-1, HLA-DR/LFA-3, or HLA-DR/DR7-1/LFA-3. To facilitate TCR engagement, the superantigen SEE was added to the cultures during stimulation. To establish the functionality of our CD40L promoter constructs, the pGL-X construct containing 1.6 kb of the CD40L promoter cloned in front of a luciferase reporter gene was transfected into Jurkat T cells, and the cells were stimulated with the different CHO transfecants described above. As a comparison, the same Jurkat cell line was transfected with an IL-2 promoter reporter construct and stimulated in parallel. The IL-2 promoter has been shown to contain DNA binding elements for the transcription factors NFAT, NF-κB, AP-1, and Oct-1 (44) and to respond to costimulatory signals in the present assay system (42). As shown in Fig. 1, transcriptional activities of both the pGL-X construct and the IL-2 promoter were induced by stimulation using CHO-DR/B7-1 transfectants in the presence of SEE. Interestingly, stimulation with CHO-DR/LFA3-3 transfected in the presence of SEE induced 50% of CD40L promoter activity compared with stimulation with DR/B7-1, whereas IL-2 promoter activity was only marginally induced by DR/LFA-3 as previously described (42). Stimulation with the triple transfecant CHO-DR/B7-1/LFA-3 in the presence of SEE resulted in transcriptional induction of the CD40L promoter to a similar level as that seen after stimulation with the CHO-DR/B7-1 transfecant. In contrast, transcriptional activation of the IL-2 promoter was superinduced using the triple CHO transfecant. Stimulation with the CHO-DR transfectant resulted in marginal induction of the IL-2 promoter, while CD40L transcription was induced about 4-fold. These data suggest that the IL-2 and CD40L promoters are differently regulated by costimulatory signals, although they share several similar transcription factor binding sites.
Induction of CD40L promoter activity in response to costimulatory signals is regulated by two distinct regions containing NFAT sites

To further define the DNA elements involved in CD40L transcription induced by costimulatory molecules we generated promoter deletants by PCR. The different CD40L promoter reporter constructs (pGL-X through pGL-TATA) are shown in Fig. 2. The 5’ deletants of the CD40L promoter were transfected into Jurkat T cells, and the transfected cells were stimulated with CHO-DR/B7-1 cells. As shown in Fig. 2, CHO-DR/B7-1 transfectants stimulated luciferase activity 12-fold above the control level of the constructs pGL-X, pGL-I, and pGL-II. The activity dropped to a 6-fold induction of the constructs pGL-III, pGL-IV, and pGL-NFAT, with pGL-IV showing a slightly higher activity than the other two constructs. This drop in induced activity corresponded to the deletion of the distal NFAT site at position -283 to -266 upstream of transcription start. The next drop in activity was seen using the pGL-TATA construct containing the TATA box only. This corresponded to deletion of the proximal NFAT site at positions -74 to -57 upstream of transcription start. These data support previous identifications of two NFAT binding sites in the CD40L promoter pertinent for functional activity (29, 30).

The proximal NFAT site is not dependent on binding of AP-1 for transcriptional activity induced by costimulatory signals

Tsytsykova et al. (30) previously showed that a pentamer of the proximal NFAT site of the murine CD40L promoter was not sufficient to drive a luciferase reporter gene when the cells were stimulated with PMA and ionomycin. Rather, this NFAT site required a flanking AP-1 sequence to be transcriptionally active. We constructed promoter deletants that contained the proximal NFAT site with and without the AP-1 site attached to it. In addition, we made a promoter deletant containing a mutated NFAT site with the AP-1 site still intact. As shown in Fig. 3, the transcriptional activity of the pGL-NFAT/AP-1 deletant was induced 7-fold compared with the control when stimulated with CHO-DR/B7-1 transfectants in the presence of SEE. Due to high background activities of the promoter constructs, we failed to obtain significant induction of promoter activities when the cells were stimulated with PMA and ionomycin (data not shown). Deletion of the AP-1 site did not affect the transcriptional activity of the construct when the cells were stimulated with CHO-DR/B7-1 transfectants in the presence
of SEE, while mutation of the proximal NFAT site reduced the induced promoter activity to background levels, although the AP-1 site was kept intact (Fig. 3). We conclude from these data that induction of transcriptional activity of the proximal NFAT site in response to signals through TCR and CD28 is not dependent upon AP-1 binding to the suggested AP-1 site.

Costimulation through CD2 and CD28 results in quantitative rather than qualitative differences in nuclear expression of NFAT proteins

The difference in magnitude of CD40L promoter activity in response to various costimulatory signals might be due to either quantitative or qualitative differences in NFAT induction and binding to the promoter. To investigate NFAT binding to the proximal site of the CD40L promoter in detail, we performed a series of EMSAs. As shown in Fig. 4A, when the proximal NFAT site was used as probe we could detect some background protein binding to the site in unstimulated cells. The binding was increased using nuclear extracts from cells stimulated with CHO-DR or CHO-DR/LFA-3 transfectants in the presence of SEE. Maximum binding to the site was found using extracts from cells stimulated with CHO-DR/B7-1 transfectants, while extracts from cells stimulated with CHO-DR/LFA-3 transfectants in the presence of SEE. As shown in Fig. 4C, addition of either Ab shifted the protein complex binding to the probe containing the proximal NFAT site in the CD40L promoter after stimulation of the cells with CHO-DR/LFA-3 transfectants as well as with CHO-DR/B7-1 transfectants in the presence of SEE. A control Ab (anti-NF-kB p50) did not shift the protein complex. This indicates that the higher CD40L promoter activity induced by stimulation through TCR/CD28 compared with stimulation through TCR/CD2 is due to increased amounts of NFAT binding to the promoter rather than induction of different NFAT components or the presence in the complex of additional transcription factors.

Induction of transcriptional activity is dependent on a region 3′ of the TATA-proximal NFAT site that contains Egr protein binding sites

As the proximal NFAT site was shown not to be dependent on the flanking AP-1 site to be transcriptionally active, we wanted to investigate whether the region downstream of the NFAT site was important for binding factors that interact with NFAT. To this end we generated a CD40L promoter deletant where the 23 bp between the NFAT site and the TATA box were substituted with a sequence that contains no detectable transcription factor binding sites (41). The promoter deletant was transfected into Jurkat T cells, and the cells were stimulated with CHO cells expressing DR/B7-1 in the presence of SEE. As shown in Fig. 5A, replacement of the 23 bp resulted in reduction of the induced transcriptional activity of the promoter construct to almost background levels. Also, when the wild-type sequence between the NFAT site and the TATA box was used as a probe in EMSA, two DNA binding protein complexes appeared in cells stimulated with CHO-DR/B7-1 transfectants (Fig. 5B). This suggests the presence of proteins binding 3′ of the TATA-proximal NFAT site that may positively influence NFAT-induced transcription.

To investigate the identity of the proteins we initially attempted to compete protein binding to the probe containing the wild-type

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**FIGURE 3.** Transcriptional activity of the proximal NFAT site of the CD40L promoter. The different NFAT constructs were generated as described in Materials and Methods. Jurkat T cells were transiently transfected with the different NFAT constructs or with pGL-TATA. After 22 h the transfected cells were stimulated in triplicate with CHO cells expressing DR/B7-1 in the presence of 100 ng/ml SEE, or they were left unstimulated. The cells were harvested 8 h after stimulation, and the luciferase activity was measured. Promoter activity for each construct is expressed as fold activation compared with transfected, but unstimulated, cells. One representative experiment of at least three performed is shown.
Discussion

Costimulation represents a diligent control system where an immune response is not initiated and amplified if several levels of control have not been passed. However, as in all cascade phenomena a given event must be limiting and by exceeding a threshold level of activation trigger downstream events that amplify the biological signal. It is well established that the CD40L is transiently expressed on predominantly CD4⁺ T cells following stimulation through the TCR. It has also been reported that costimulation through CD28 can further induce and stabilize CD40L expression (12, 17–19). We wanted to investigate the regulation of CD40L promoter activity following stimulation through TCR and costimulatory molecules, and we used a system where both signals are present on the same cell, i.e., the CHO transfectants. We first show that the IL-2 promoter and the CD40L promoter are distinctly regulated in response to costimulatory signals. Although transcription of the CD40L gene is induced by TCR ligation alone and further up-regulated by signaling through costimulatory molecules, transcription of the IL-2 gene is not induced until the T cells are fully activated by the presence of both B7 and LFA-3 interactions. This reflects the fact that while CD40L functions as a costimulatory molecule acting early during immune triggering, IL-2 is a cytokine that acts downstream, and once secreted it will trigger several cell types independently of their clonal specificity. Hence, we envision a scenario where low levels of transcription and expression of CD40L is triggered by TCR stimulus alone or together with the constitutive costimulatory molecule CD2. This CD40L expression will induce expression of other costimulatory molecules if the proper cell type is taking part in the interaction. The expression of other costimulatory molecules, such as B7, will induce even higher levels of CD40L expression and trigger an amplification cascade of positive signals. It is implicit in our scenario that CD40L in a given event must be limiting and by exceeding a threshold level of activation trigger downstream events that amplify the biological signal. Thus, we synthesized an oligonucleotide containing both the proximal NFAT site and the potential Egr binding site. When this oligonucleotide was used as a probe in EMSA together with nuclear extracts from Jurkat cells stimulated with CHO-DR/B7-1 transfectants, three bands appeared (Fig. 5C). To identify the proteins binding in the different complexes we performed supershifts using Abs toward NFATp, NFATc, Egr-1, Egr-2, and Egr-3 as well as a control Ab (anti-NF-κB p65). As shown in Fig. 5C, the upper complex was supershifted using Abs toward NFATp and NFATc, but also using Abs toward Egr-1 and possibly Egr-3. Abs toward Egr-2 or p65 did not shift the complex. In conclusion, our data suggest that in response to signals through TCR and CD28, Egr proteins bind to a newly identified site in the CD40L promoter located just downstream of the proximal NFAT site.

We also investigated the CD40L promoter in detail by generating 5’ promoter deletants. By transfecting these deletants into Jurkat T cells we could identify two areas, both containing NFAT binding sites, that are required for promoter activity in response to costimulatory signals (Fig. 2). Analysis of the TATA-proximal 23-bp sequence 3’ of the NFAT site in EMSA. The complexes were competed for binding when an excess of unlabeled self oligonucleotide was used, but we did not manage to reproducibly compete binding with either oligonucleotides derived from the IL-2 NFAT site or the consensus binding sequences of Egr, NF-κB, AP-1, and Oct (data not shown). However, based on sequence similarities we suspected that proteins of the Egr family might bind to the 5’ region of the sequence and that binding conditions thus may be suboptimal. Therefore, we synthesized an oligonucleotide containing both the proximal NFAT site and the potential Egr binding site. When this oligonucleotide was used as a probe in EMSA together with nuclear extracts from Jurkat cells stimulated with CHO-DR/B7-1 transfectants, three bands appeared (Fig. 5C). To identify the proteins binding in the different complexes we performed supershifts using Abs toward NFATp, NFATc, Egr-1, Egr-2, and Egr-3 as well as a control Ab (anti-NF-κB p65). As shown in Fig. 5C, the upper complex was supershifted using Abs toward NFATp and NFATc, but also using Abs toward Egr-1 and possibly Egr-3. Abs toward Egr-2 or p65 did not shift the complex. In conclusion, our data suggest that in response to signals through TCR and CD28, Egr proteins bind to a newly identified site in the CD40L promoter located just downstream of the proximal NFAT site.

FIGURE 4. EMSA showing nuclear expression of NFAT after stimulation with different CHO transfectants. A, Nuclear extracts from unstimulated Jurkat cells and cells stimulated with the different CHO cells or with PMA/ionomycin as described in Materials and Methods were incubated with radiolabeled oligonucleotide containing the proximal NFAT site of the CD40L promoter. The radiolabeled Oct oligonucleotide was used as a control for protein content in the nuclear extracts. B, Cross-competition experiments using nuclear extracts from cells stimulated with CHO-DR/B7-1 in the presence of SEE. Protein binding was competed for by unlabeled oligonucleotides containing the proximal CD40L NFAT site (self), CD40L NFAT extended, CD40L NFAT extended mutatod, IL-2 NFAT (ARRE-2), or TRD. The sequences of the different competitors are described in Materials and Methods. C, Supershift analysis of NFAT binding complexes using nuclear extracts from cells stimulated with CHO-DR/LFA-3 or CHO-DR/B7-1 transfectants in the presence of SEE or PMA/ionomycin. Nuclear extracts were incubated with the radiolabeled CD40L NFAT oligonucleotide in the presence or the absence of Abs to NFATp or NFATc or a control Ab. One representative experiment of at least three performed is shown.
NFAT site showed that it is not dependent on the flanking AP-1 site, as proposed by Tsytsykova et al. (30) (Fig. 3). However, there are several differences between our study and the previous one. In the study performed by Tsytsykova et al., a construct containing pentamers of the proximal NFAT site cloned in front of the SV40 minimal promoter was shown to be transcriptionally inactive without the AP-1 site attached to it. In the present study, we used the original promoter sequence with the NFAT site in the exact position upstream of the endogenous TATA box. In addition, Tsytsykova et al. used a murine T cell hybridoma, while we transfected the murine promoter into a human Jurkat T cell line (it should be noted that the murine and the human CD40L promoter are fully conserved in the sequences containing the NFAT and the AP-1 site investigated). Lastly, in the previous study, the cells were stimulated with PMA and ionomycin. We failed to obtain significant induction of transcriptional activity of the CD40L promoter when Jurkat cells were stimulated with PMA and ionomycin due to high background activities of the constructs. Instead, we used CHO transfectants that expressed HLA-DR and the costimulatory molecules B7-1 and LFA-3 on the same cell. Stimulation using these transfectants will result in ligation to TCR as well as to CD28 or CD2, and the cells will receive the endogenous intracellular signals from these receptors, which we believe represents a more balanced stimulation procedure.

Signals through TCR induce the activation of protein tyrosine kinases that phosphorylate numerous downstream substrates (45), having as an end point the activation of several transcription factors, such as NFAT and NF-κB. Costimulation through CD28 also require protein tyrosine kinases (46), which subsequently lead to activation of AP-1 and NF-κB (47, 48) as well as NFAT (25). Costimulation through CD28 has been shown to induce activation of NFAT (26, 49) as well as the mitogen-activated protein kinases extracellular-regulated kinase and c-Jun N-terminal kinase (49). As shown in Fig. 1, stimulation through TCR and CD28 results in a higher induction of CD40L promoter activity than stimulation through TCR and CD2, which might be due to either qualitative or quantitative differences in the composition or activation of induced transcription factors. As shown in Fig. 4A, stimulation through TCR/CD28 results in a more dramatic increase in nuclear NFAT expression than stimulation through TCR/CD2, suggesting that the higher CD40L promoter activity in response to signals through TCR/CD28 is due to increased nuclear expression of NFAT rather than to induction of additional transcription factors. Supershift analysis of nuclear extracts from cells stimulated through TCR/CD28 also showed similar composition of NFAT subunit binding to the proximal NFAT site of the promoter regardless of stimulation (Fig. 4C). NFAT most often requires interactions with additional transcription factors to be active, one of them being AP-1. In the murine IL-2 promoter NFAT interacts with AP-1 in four of five binding sites (27). However, the human Fas (CD95) ligand promoter has been shown to contain two NFAT binding sites that function independently of AP-1 binding (50, 51). The Fas ligand and CD40L belong to the same family of proteins, the TNF family, and both molecules are induced early after TCR ligation. Studies of the NFATp knockout mouse, show defects in early Fas ligand and
CD40L expression (52), suggesting that expression of these molecules may be regulated in similar ways. Our data support this theory, because we show that the proximal NFAT site of the CD40L promoter is transcriptionally active without adjacent AP-1 binding. However, the Fas ligand promoter contains binding sites for AP-1 as well as for additional transcription factors, including NF-κB, but their respective binding sites are not located close to the defined NFAT sites (53, 54).

To investigate putative transcription factors binding 3′ of the NFAT site in the CD40L promoter we focused on the 23-bp region between the proximal NFAT site and the TATA box. We substituted the wild-type sequence with a sequence that contains no transcription factor binding sites. The transcriptional activity of the 3′ deletant construct was markedly reduced compared with the NFAT construct when the cells were stimulated through TCR/CD28 (Fig. 5A). When the wild-type sequence was used as a probe in EMSA, two weak bands appeared upon stimulation of the Jurkat cells with the construct when the cells were stimulated through TCR/CD28 (Fig. 4). This suggests that the proximal NFAT site of the CD40L promoter is 5′-GTGTGGGTT-3′, and that in the human promoter 5′-GTGTGGGCT-3′. The sequence similarities and the competition experiments together suggest that Egr proteins may bind to this region. However, because the sequence is located at the very end of the 23-bp region, it may result in inadequate binding conditions for the proteins. Therefore, we generated an oligonucleotide containing the proximal NFAT site as well as the suspected Egr binding site (positions −74 to −45). When this oligonucleotide was used in EMSA together with nuclear extracts from CHO-DR/β7-1 transfected Jurkat cells, three bands appeared (Fig. 5C). Supershift assays revealed that the upper complex contained NFATc and NFATp as well as Egr-1 and possibly Egr-3 (Fig. 5C). It has recently been shown that both the Egr-1 and Egr-3 proteins bind to the same region (FLRE) in the human Fas ligand promoter, but only Egr-3 was shown to induce transcriptional activity (55). However, another study of the human Fas ligand promoter showed that Egr-1 and Egr-3 bind together with NFAT on two sites and cooperate with NFAT to induce transcription on three sites in the promoter (56).

We do not currently know whether both Egr-1 and Egr-3 are involved in transcriptional regulation of the CD40L promoter or whether cooperation with NFAT is required. However, mutation of the proximal NFAT site as well as deletion of the Egr site reduced the induced transcriptional activity to background levels, suggesting a cooperation between the two transcription factors. Supershift assays also revealed that the NFAT proteins and the Egr proteins bind together in a complex, as previously shown in the Fas ligand promoter (56). This supports the idea that the CD40L promoter is regulated similarly to the Fas ligand promoter where binding of NFAT and Egr-3 (and perhaps Egr-1), but not AP-1, has been shown to be important for transcriptional activity induced by signals through TCR.

In conclusion, we have described the induction of CD40L promoter activity in response to signals through TCR and CD28 to be dependent on binding of NFAT to two sites in the promoter, where the proximal NFAT site is not dependent on AP-1 binding. We have also identified a previously unknown Egr binding site located just downstream of the proximal NFAT site. We are currently investigating the role of the Egr proteins in regulation of transcriptional activities of the CD40L promoter.

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