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*J Immunol* 2000; 165:263-270; doi: 10.4049/jimmunol.165.1.263

http://www.jimmunol.org/content/165/1/263
The HTLV-I Tax Protein Transcriptionally Modulates OX40 Antigen Expression

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OX40 is a member of the TNF receptor family, expressed on activated T cells. It is the only costimulatory T cell molecule known to be specifically up-regulated in human T cell leukemia virus type-I (HTLV-I)-producing cells. In a T cell line, OX40 surface expression was shown to be induced by HTLV-I Tax alone. To understand molecular mechanisms of OX40 gene regulation and modulation by HTLV-I Tax, we have cloned the human OX40 gene and analyzed its 5′-flanking region. By reporter gene analysis with progressive 5′ deletions from nucleotides −1259 to −64, we have defined a 157-bp DNA fragment as a minimal promoter for constitutive expression. In addition, we show that in the OX40⁺ cell line, Co, Tax is able to further increase OX40 surface expression. Up-regulation of OX40 promoter activity by Tax requires two upstream NF-κB sites, which are not active in the constitutive OX40 expression. Their deletion abrogates Tax responsiveness in reporter gene analysis. The site-directed mutagenesis of each NF-κB site demonstrates that cooperative NF-κB binding is a prerequisite for Tax-directed activity as neither site alone is sufficient for a full Tax responsiveness of the OX40 promoter. Upon Tax expression, both sites bind p65 and c-Rel. These data provide new insight into the direct regulation of OX40 by Tax and add to our understanding of the possible role of the OX40/OX40 ligand system in the proliferation of HTLV-I⁺ T cells. The Journal of Immunology, 2000, 165: 263–270.

The T cell costimulatory receptor OX40 and its ligand (OX40L)² are both members of the superfamilies of TNF receptors and ligands, respectively (1–4). Interactions of these members with their ligands have been shown to be involved in cell proliferation, activation, as well as induction of apoptosis. However, unlike the other known TNF receptor family members, OX40 has a restricted cellular expression only on activated lymphocytes, predominantly CD4⁺ T cells (3, 5, 6). Furthermore, it has thus far not been associated with a cell death-inducing function. Rather, signaling through OX40 generates strong costimulatory effects, which induce T cell proliferation (7, 8), modulate cytokine production (9), and influence T cell migration into tissues (10, 11). Both OX40 and OX40L are constitutively expressed on human T cell leukemia virus type I (HTLV-I)-producing T cell lines (3, 10–12).

HTLV-I infection is associated with the aggressive and lethal adult T cell leukemia (ATL), as well as chronic inflammatory disorders, such as the tropical spastic paraparesis/HTLV-I-associated myelopathy and others (13–15). T cell transformation requires the HTLV-I Tax oncoprotein, which is the activator of viral gene expression. Tax also acts as a transactivator of an increasing number of host cellular genes, most of which are associated with cell growth. They include growth factors, like GM-CSF (16) or cytokines and their receptors such as IL-2 (17), IL-15 (18), or IL-2Rα (CD25) (19–21). This activation occurs via the interaction of Tax with cellular transcription factors, which include the serum response factor (22, 23), members of the serum response factor/cAMP response element binding protein (24, 25), and NF-κB (26).

NF-κB defines a family of transcription factors that result from the combination of its protein members, such as p50, p65 (RelA), c-Rel, and RelB (27). Inactive NF-κB is retained in the cytoplasm, either by complexing with the IκB inhibitor or as a precursor molecule of the active protein (27). NF-κB activation and subsequent translocation to the nucleus requires phosphorylation and degradation of IκB or proteolytic cleavage of the precursor molecule. Tax is one of the signals known to cause liberation of NF-κB from IκB (26, 28). It can also bind NF-κB precursors as well as active nuclear NF-κB proteins (23, 29–31).

The OX40L promoter was shown to be activated by the Tax oncoprotein (32) and expression of Tax in the OX40-negative T cell line, Jurkat, results in OX40 surface expression (33). These findings suggest that auto or paracrine OX40/OX40L interactions on HTLV-I-producing cells may provide necessary costimulatory signals for transformation or survival and proliferation of these cells. Thus far, however, little is known about the transcriptional regulation of OX40 gene expression and its modulation by the Tax protein. Therefore, we have cloned the human OX40 gene, analyzed its promoter region, and defined basal promoter activity. Moreover, we describe here that Tax further up-regulates OX40 gene expression in an OX40⁺ cell line via two NF-κB elements, which recruit at least three members of the NF-κB family: c-Rel, p65, and p50 in a Tax-dependent manner.

Materials and Methods

Isolation and characterization of human OX40 genomic clones

The human placental cosmid library pWE15 (Stratagene, Heidelberg, Germany) was screened by standard molecular biology techniques using random-primed radio-labeled human OX40 cDNA (3). The cosmid carrying
of each plasmid DNA. Cell extract preparation and luciferase assay were performed 48 h later according to the Luciferase assay system kit (Promega). Enzyme activity was measured with a Lumat LB9501 (Berthold, Wildbad, Germany). Transfection efficiency was normalized by cotransfection of a reporter plasmid, pCH101 (Pharmacia Biotech, Freiburg, Germany), containing the β-galactosidase gene under the control of the SV40 promoter. β-galactosidase activity was determined using the Galacto-light kit (Tropix, Bedford, MA), following manufacturer’s instructions. For comparison of promoter activities in different cell lines, luciferase activities were referred to luciferase control vector, pGL2 control, containing the SV40 promoter and enhancer. All measurements were conducted in duplicates from three independent transfections.

Nuclear extracts and gel mobility shift assay

Nuclear extracts were prepared as previously described (37). Five micrograms of extract were incubated with 1 ng of labeled DNA (20,000 cpm) in a buffer containing 20 mM HEPES (pH 7.9), 60 mM KCl, 0.5 mM dithiotreitol, 4% Ficoll or 1% polyvinylalcohol, 5% glycerol, and 2 μg poly (dl-dC) (Boehringer Mannheim) for 30 min at 4°C. The following oligonucleotides with consensus sites underlined and mutations in lower case letters were used as probes: NF-κB site 1, 5′-ACGGCTGGGAATTCG CACAGGTGTTG-3′ (-1182/-1156); NF-κB site 2, 5′-TCTGGGAG GAGGGGATTTCCAGCGGC-3′ (-1128/-1102); NF-κBmut, 5′-GATG GATGATGGAGGACGTTTCCCTCTTTACT-3′. Oligonucleotides carrying an Sp-1 site, Sp-1cons, 5′-ATTCGATCGGGGCGGGGCGGC-3′ were purchased from Santa Cruz (Heidelberg, Germany). The reaction mixtures were electrophoresed through a 4% polyacrylamide gel. In competition experiments a 50-fold excess of unlabeled oligonucleotides, and in supershift assays 2 μg of NF-κB p50 (sc-1191X), NF-κB p65 (sc-372X), or NF-κB c-Rel (sc-70-GX) Abs (Santa Cruz), were added to the binding reaction before the addition of the labeled probe.

Results

Analysis of the human OX40 gene

To determine the genomic structure of the OX40 Ag, a cosmid containing the entire OX40 gene was obtained. The complete coding region was found to be within only 2.7 kb and to include 7 exons and 6 introns (DDBJ/EMBL/GenBank Nucleotide Sequence Database accession number AJ277151). To identify the transcription initiation site, we performed RTase protection assays with RNA isolated from OX40⁺ HUT-102 cells. We obtained a protected band that mapped the start site to an adenosine 26 nt upstream of the ATG initiation codon (Fig. 1A). The specificity of the transcription start site was confirmed using RNA of a different OX40⁺ cell line, MT-2 (data not shown).

Fig. 1B depicts the 5′-flanking region and putative transcription factor binding sites, based on sequence homology. Although an initiator element (Inr) matching the consensus sequence Py Py A to +1 N T/A Py Py (38) overlapped the start site for transcription, the sequence analysis additionally revealed a TATA-like box at position −27 as well as a CCAAT box. Several elements with homology to Sp-1 binding sites, such as CCCCCTCC (39) or GT box sequences (40), were found throughout the 5′ region. Furthermore, we found two putative E boxes, as well as potential binding sites for AP-1, AP-2, Yin Yang-1, and Ikaros-2. Finally, at the very 5′-end of the analyzed region two NF-κB binding sites were detected (Fig. 1B).

Functional analysis of the human OX40 promoter region

First, to test the promoter activity of the 5′-flanking sequence of the OX40 gene, a fragment spanning the region between nucleotides −1259 to +19 was cloned upstream of the luciferase gene of the pGL2 basic vector. This reporter construct was then transiently transfected into OX40⁺ DG75 and L63 cells, and into Co and CCRF-CEM cells, which both constitutively express low levels of OX40 as determined by FACS analysis (Fig. 2). We compared the ability of the OX40 5′ fragment to induce luciferase activity in these cell lines.
Indeed, luciferase activities in Co and CCRF-CEM cells were 20 times higher than in OX40 DG75 and L363 cells (Fig. 3A). The OX40 promoter in the OX40 cells was three times less active than the SV40 promoter and enhancer of the pGL2 control vector. This showed that the sequence up to −1259 serves as a promoter of OX40 gene expression in the OX40 cell lines.

To determine cis-acting elements in the 5′-flanking region, which regulate OX40 gene expression, we transfected Co and CCRF-CEM cells with a series of luciferase reporter constructs. Starting from position −1259, we progressively deleted the 5′-end, to position −1021, −944, −649, −426, −2138, and −64. As shown in Fig. 3B, all constructs displayed a reduced luciferase activity compared with the −944 construct, which was the most potent one and therefore set as 100%. The longest construct −1259 displayed a 2.5-fold lower activity than the −944 construct. Deleting the region to −1021 resulted in an increased promoter activity being only 1.4-fold lower than the −944 construct. However, as neither Co (see Fig. 5B) nor CCRF-CEM cells (data not shown) exhibit nuclear NF-κB activity the NF-κB sites within this region do not play a role in maintaining the constitutive OX40 promoter expression in these cells.

To further define the minimal promoter elements required for OX40 expression, other 5′ deletion constructs were tested. Deletions from nucleotide −944 to −649 and to −426 resulted in 2- and 2.5-fold lower promoter activities, respectively (Fig. 3B). Remarkably, the promoter activity of the −138 construct was only 1.4-fold lower than the −944 construct. Indeed, this sequence lacks the CCAAT element but contains the TATA element and several Sp-1 binding sites, which were found to bind both Sp-1 and Sp-3 in EMSA (data not shown). A further 5′ truncation to nucleotide −64 that deleted the Sp-1 site located at position −118, diminished the promoter activity 5-fold compared with the −944 construct, but still maintaining it above background levels. Thus these data demonstrate that the region between −138 and +19 can serve as a minimal promoter whose activity seems to be dependent on the number of Sp-1 sites present.

**Tax modulates OX40 promoter activity**

To determine whether Tax is able to further up-regulate OX40 Ag expression, we transfected Co cells with Tax expression plasmids: wild-type Tax (pSGtax) or, as a negative control, a truncated Tax (pSGtaxmutant2) lacking amino acids 285–353, which was shown to have lost its transactivation ability (36, 41). FACS analysis demonstrated that OX40 expression could be up-regulated only upon wild-type Tax and not upon transfection with the pSGtaxmutant2 plasmid, as shown in Fig. 4A. Thus, functional Tax expression increases OX40 surface expression.

To examine the role of the OX40 5′-flanking region in Tax-induced OX40 up-regulation, we cotransfected Co cells with the −1259 OX40 promoter construct together with the pSGtax or with the pSGtaxmutant2 plasmids. Coexpression of the truncated Tax protein (pSGtaxmutant2) did not affect reporter gene expression in
any of the OX40 promoter constructs (Fig. 4B). However, cotransfection of the −1259 construct with pSGtax was associated with a 6-fold increase over control in luciferase activity. Thus Tax expression indeed induced OX40 promoter activity.

To localize the promoter region responsive to Tax, the 5′-end deletion constructs, −1021, −944, and −138, were employed for cotransfection. In fact, the deletion of the region between −1259 and −1021, including two Sp-1 sites and NF-κB sites 1 and 2, fully abolished Tax-induced promoter activity (Fig. 4B). In addition, Tax expression did not alter the activity of the minimal promoter as evidenced with the −138 construct. These results indicate that the minimal promoter of the OX40 gene is Tax independent and that the upstream region containing Sp-1 and two NF-κB sites accounts for the promoter responsiveness upon Tax.

Two NF-κB sites are required for Tax inducibility

We wondered whether the two NF-κB sites found within the −1259 and −1021 region might mediate OX40 promoter inducibility upon Tax. Therefore, a series of reporter constructs carrying mutations within either NF-κB site was generated (Fig. 5A). To exclude that any relevant binding to the sequence upstream of both NF-κB sites could influence Tax modulation, we generated construct −1184 deleting the region between nucleotides −1259 and −1184. To this construct we then introduced point mutations either to the NF-κB site 1, −1184 mut1, or to the NF-κB site 2, −1184 mut2. Furthermore, construct −1124 mut2 lacked the NF-κB site 1 and contained a mutated NF-κB site 2. These constructs were transfected into Co cells in the presence or absence of pSGtax cotransfection. In the absence of pSGtax cotransfection, all constructs exhibited promoter activities equivalent to the −944 construct. As expected, point mutations in either NF-κB site did not affect the constitutive promoter activity. This is consistent with our findings in EMSAs where nuclear NF-κB activity is absent in Co cells (Fig. 5B). Furthermore the point mutations within NF-κB site 2 mutated the overlapping Sp-1 site as well, with no effect on the resulting promoter activity. However, when we cotransfected pSGtax we detected a >5-fold increase of the activity in the nonmutated −1184 construct. The respective point mutations of either NF-κB site severely reduced the inducibility of luciferase activity by Tax to <2-fold (Fig. 5A). Finally, the deletion of NF-κB site 1 and a mutation of NF-κB site 2 (−1124 mut2) completely abolished OX40 promoter inducibility upon Tax coexpression. Therefore, the NF-κB

FIGURE 3. A, OX40 promoter-directed reporter gene activity. Co and CCRF-CEM (OX40+) and DG75 and L363 (OX40−) cell lines were transfected with 1 μg of the pGL2 basic −1259 to +19 OX40 construct. After a 48-h incubation, cells were harvested and assayed for luciferase activity. The luciferase activities were measured in duplicates, normalized to β-galactosidase activity, and referred to the activity of the pGL2 control vector (set 100,000 random light units). The means of three independent experiments are shown with SDs given on top of each bar. B, 5′-deletion analysis of the OX40 promoter. Co (□) and CCRF-CEM (■) cells were transfected with a series of pGL2 basic constructs containing 5′-terminus promoter deletions, as indicated on the abscissa. Luciferase activities were measured in duplicates and normalized to β-galactosidase activity. The mean of three independent experiments is shown as a percentage of the activity of the −944 construct (100%) with SDs given on top of each bar.
sus oligonucleotides (Fig. 5). In fact, Sp-1 binding could be specifically competed by Sp-1 consensus sites in nuclear extracts from untransfected Co cells, which served as controls. To determine whether NF-κB complexes bind to NF-κB site 1 and 2 upon Tax transactivation, we performed EMSAs using the two NF-κB sites, although not required for a constitutive OX40 expression, play a central role in OX40 promoter regulation upon Tax expression. Furthermore, both sites are required for optimal Tax-induced OX40 promoter activity. Furthermore, we show that Tax can enhance OX40 constitutive expression with several Sp-1 binding sites modulating promoter activity. Tax-induced promoter modulation requires a 79-bp sequence located 1.1 kb upstream in the OX40 enhancer region. The cooperative function of two distinct NF-κB sites within this sequence is shown here to be the prerequisite to confer Tax responsiveness by binding nuclear p50, p65, and c-Rel.

Examining the OX40 gene promoter, we have determined that transcription could be initiated by an Inr element and a TATA box. Inr elements are often found in TATA-less promoters because they can replace the TATA box to direct the precise transcription start (44). However, when Inr and a TATA box are present in the same core promoter with the TATA box 25–30 bp upstream of the Inr, they can cooperate to enhance promoter activity, even if the TATA box is weakly competed with unlabeled Sp-1 oligonucleotides, as they overlaid the anti-p50 supershift (Fig. 5C). Taken together, these results suggest that p65, c-Rel, and, to a lesser extent, p50-binding to the NF-κB site 1 and site 2 are responsible for mediating Tax-induced OX40 promoter up-regulation.

Discussion

Signaling through OX40 provides a potent costimulatory function for prolonging the life span of T cells. The OX40 Ag is also constitutively expressed upon HTLV-I infection, thus possibly contributing to the survival and proliferation of the infected cells. In view of the importance to understand the regulatory mechanisms that control OX40 expression, the cloning of the gene and the characterization of mechanisms involved in its promoter regulation described here contribute a substantial advance in OX40 biology. We define a 157-bp DNA fragment as a minimal promoter for constitutive expression with several Sp-1 binding sites modulating its activity. Furthermore, we show that Tax can enhance OX40 surface expression and that it can transcriptionally up-regulate the promoter activity. Tax-induced promoter modulation requires a 79-bp sequence located 1.1 kb upstream in the OX40 enhancer region. The cooperative function of two distinct NF-κB sites within this sequence is shown here to be the prerequisite to confer Tax responsiveness by binding nuclear p50, p65, and c-Rel.

Previously, it has been reported that OX40 Ag expression is induced by the viral transcriptional activator HTLV-I Tax (33). Here we show that Tax enhances OX40 expression in an OX40+ cell line. In fact, this suggests that Tax is not only capable to induce de novo gene expression, but can also modify cellular Ag
expression to meet the requirements for viral persistence. On the promoter regulatory level, this modified OX40 gene expression upon Tax relies on the presence of two NF-κB sites spanning a region of 79 bp in the enhancer region. These sites are sufficient for conferring the Tax responsiveness of the whole OX40 5' flanking region, examined in Fig. 5A. They are dispensable in the constitutive expression of the OX40 gene, as untransfected Co cells do not exhibit any nuclear NF-κB binding activity (Fig. 5B).

Upon Tax expression, both OX40 NF-κB sites bind the same set of NF-κB complexes with equal strength (Fig. 5B). These complexes consist of mainly p65 and c-Rel (Fig. 5B). Other cellular promoters have also been shown to be up-regulated by Tax through NF-κB proteins, such as the IL-2Rα (19) or OX40L (32).

The NF-κB site in the IL-2Rα promoter, GGGAATCTCC, differs from the OX40 NF-κB site 1 in one and from site 2 in only two positions of the decameric motif. Like OX40, the IL-2Rα NF-κB site has also been shown to bind p65 and c-Rel upon Tax expression, with c-Rel serving as the major protein responsible for Tax transactivation (19). Interestingly, one NF-κB site in the IL-2Rα promoter is sufficient to mediate Tax-directed transcriptional up-regulation, whereas in the OX40 promoter two cooperatively acting NF-κB sites are required (Fig. 5A). However, two NF-κB elements, i.e., GGGAATTCA and GGGAACCTTCT, have also been described to be required for Tax transactivation in the OX40L promoter. Upon Tax expression, no binding of c-Rel but rather of RelB was detected on these sites (32). The respective NF-κB

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**FIGURE 5.** A, Functional role of two NF-κB sites for OX40 promoter inducibility by Tax. The pGL2 basic constructs carrying the OX40 −1184 to +19 fragment with wild-type NF-κB sites (−1184), a mutated NF-κB site 1 (−1184 mut1, GGGAATTCC → GGCAATTTCC), a mutated NF-κB site 2 (−1184 mut2, AGGGATTTCC → AGCCATTTCC), or a construct spanning the −1124 to +19 region and carrying the mutated NF-κB site 2 (−1124 mut2) were transfected into Co cells in the absence or presence of cotransfected pSGtax. Luciferase activities of the NF-κB/OX40 promoter constructs in the absence of cotransfected pSGtax vector are shown as a percentage of the activity of the −944 construct (100%), with the activity of the −1021 construct included (left diagram). The Tax-dependent increase of luciferase activity of the NF-κB/OX40 promoter constructs upon cotransfected pSGtax is shown in the right diagram. Luciferase activities were determined in duplicates and normalized to β-galactosidase activity. Shown is the mean of three independent experiments with SDs given on top of each bar. B, Binding of nuclear proteins to the OX40 NF-κB sites. 32P-labeled oligonucleotides containing the NF-κB site 1 or the NF-κB site 2 were assayed for binding by Co, Co tax (Co cells, 48 h transiently transfected with pSGtax), and HUT-102 nuclear extracts. NF-κB complexes are indicated as I and II and Sp-1 complexes by arrows. To assay binding specificity, unlabeled duplex oligonucleotides for NF-κB site 1 or 2, mutated NF-κB (NF-κBmut), or Sp-1 consensus (Sp-1cons) were used as competitors at a 50-fold molar excess. C, Supershift analysis of the NF-κB family protein members binding to the OX40 NF-κB sites. Supershift experiments were performed by preincubation of HUT-102 nuclear extracts with c-Rel, p65, or p50 Abs, before addition of the 32P-labeled NF-κB probes. The respective supershifted bands are indicated by arrows. For a better resolution of the data, complexes binding to the NF-κB site 2 probe were analyzed in the presence of a 50-fold molar excess of unlabeled duplex Sp-1 competitor.
motifs in the OX40 and OX40L promoters differ in at least two or three positions from each other. Thus, the exact NF-kB motif in a given promoter may account for altered binding affinities for NF-kB proteins, and how NF-kB proteins regulate a cellular promoter in response to Tax may depend on both the sequence of the NF-kB motif and the number of sites present.

The fact that OX40 is constitutively expressed on HTLV-I-expressing T cell lines and can be detected in ATL cells could account for some of the observations reported for ATL or its related diseases (14, 15). For example, OX40 expressed on leukemic cells isolated from peripheral blood of ATL patients was shown to mediate cell adhesion to OX40L-expressing vascular endothelial cells (11), suggesting the involvement of OX40 for leukemic cell migration into tissues. Studies on normal T cells have demonstrated that OX40 is a potent costimulatory molecule, which leads to a sustained proliferation of differentiated effector T cells (8). In addition, OX40 has not been shown so far to promote cell death. Recently, reported data rather suggest that signaling through OX40 and its ligand, OX40L (4, 32), may serve to inhibit activation-induced T cell death (47). This could be important for supporting the survival of HTLV-I-expressing T cells as Tax not only guarantees for constitutive OX40 expression, but also of its ligand, OX40L (4, 32). Therefore, auto or trans activation of the OX40 promoter is expected to result in a transcriptionally active factor that binds to the OX40 promoter and mediates the expression of the OX40 gene. This is consistent with the observation that constitutively expressed OX4034 can be detected in ATL cells and can be repressed by the Tax protein.

A number of studies have shown that OX40L is a target of the HTLV-I Tax protein. Indeed, Tax protein can induce the expression of OX40L (4, 32), and OX40L expression is increased in Tax-expressing cell lines and can be detected in ATL cells could account for the possible involvement of OX40 in leukemic cell infiltration. Blood 89: 2951.


