Involvement of Jun and Rel Proteins in Up-regulation of Interleukin-4 Gene Activity by the T Cell Accessory Molecule CD28*

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CD28 serves as a costimulatory cell surface molecule in T cell activation. CD28 signaling may also play a role in balancing the inflammatory/humoral (Th1/Th2) responses during an immune reaction. CD28 costimulation has been shown to promote the production of Th2 cytokines including interleukin (IL)-4, a key cytokine essential for Th2 differentiation and for the pathogenesis of allergic inflammation. In this study, we show that IL-4 mRNA and activity of the IL-4 promoter can be activated by the CD28 signal alone and are further augmented by CD28 costimulation of α-CD3- or mitogen-activated Jurkat T cells. Two important IL-4 enhancer elements, positive regulatory element (PRE)-I and P1, are found to respond to CD28 stimulation-induced transactivation. In contrast to the Th1 IL-2 CD28RE, activity of the IL-4 PRE-I and P1 can be induced by the CD28 signal alone. In correlation with CD28-induced transcriptional activation, AP-1 (c-Jun, JunD) and NF-xB/Rel (c-Rel, RelA) family members are found to bind to the two regulatory elements PRE-I and P1 upon CD28 stimulation. The data provide the first mapping of the CD28-responsive site in a Th2 cytokine gene, the IL-4 gene. They also show that the CD28 signal can directly activate a gene (e.g. IL-4) at the transcriptional level.

Activation of T cells during an immune response requires two distinct signals: one from the engagement of the T cell receptor (TCR)‡ with specific major histocompatibility complex antigens presenting peptides on antigen-presenting cells and a second signal originating from other cell surface receptors/ligands that mediate costimulation (1). Work over the last several years has demonstrated that the CD28 glycoprotein, expressed on the surface of 80% of human T cells and on virtually all murine T cells, is one of the major costimulatory molecules (2, 3). Ligation of CD28 with anti-CD28 (α-CD28) monoclonal antibodies (mAb) or cells expressing CD28 ligands (B7-1 and B7-2) in combination with limited concentrations of anti-CD3 (α-CD3) or antigen promotes cell cycle progression and increases interleukin (IL)-2 production by regulating IL-2 mRNA at both the level of transcription and translation (for a review, see Ref. 4). Thus, CD28 can deliver biochemical signals that function in synergy with TCR-mediated signaling to initiate and maintain T cell responses.

Recent findings indicate that CD28 ligation acts not only synergistically with TCR ligation to maximize T cell signaling but also promotes cell differentiation. Several studies both in mice (5–8) and humans (9–11) support a fundamental role for CD28 in the early differentiation of T helper (Th) subsets toward a Th2 phenotype. In the absence of CD28 signaling, naive T cells are biased toward a Th1 phenotype. Th2 cells, which produce IL-4, -5, -6, -10, and -13, regulate humoral immune responses and Th1 cells, which secrete IL-2, tumor necrosis factor-β, and interferon-γ, are involved in cell-mediated and delayed type hypersensitivity immune responses. Thus, CD28 signaling may regulate the balance of inflammatory/humoral (Th1/Th2) responses during an immune reaction.

It has been shown that CD28 costimulation may directly produce the growth of Th2 cytokines (12). Both in humans (9) and in mice (12), increased CD28 ligation resulted in increased production of IL-4 and IL-5. Purified naive human T cells stimulated with α-CD3 in the absence of CD28 costimulation produce only IL-2 and interferon-γ, whereas the addition of α-CD28 mAb induced IL-4 (9). IL-4 is a pleiotropic cytokine with a wide range of biological effects on many hemopoietic and nonhemopoietically derived cells and tissues (13). IL-4 is also a well known cytokine that plays a central role in the pathogenesis of allergic inflammation by promoting Ig class switching to IgE (13). In recent years, IL-4 was also shown to be the major determining factor in the differentiation of naive T cells into the Th2 phenotype and facilitating the humoral immune response (6, 14). In the absence of IL-4, Th cells develop mainly into the Th1 phenotype (15, 16). Up-regulation of the IL-4 production by CD28 signaling may be one of the mechanisms that promotes Th2 differentiation.

The effect of CD28 on transcriptional activity of the Th1 IL-2 gene has been intensively studied. A NF-xB-like sequence between −164 and −154 and an adjacent AP-1 site of the IL-2 promoter was found to respond to the CD28 signal (17–20). NF-xB/Rel and AP-1 transcription factors were found to participate in the formation of the CD28-responsive element-specific complex and to be involved in CD28-stimulated transactivation (20–23). In addition, several IL-2 NF-AT sites were found to respond to CD28 costimulation. CD28 inducibility was shown to be conferred by the AP-1 component in NF-AT/AP-1 composite elements (24).

Although the CD28-responsive element of the IL-2 gene has been well studied, the CD28 response element of the IL-4 gene has not been characterized. Like the IL-2 gene, the IL-4 gene is regulated by multiple regulatory elements (for a review see Ref. 25). In this study, we provide the first report of CD28-responsive elements (CD28REs) in the IL-4 promoter. We show that two IL-4 promoter elements, PRE-I and P1, are responsive to the CD28 stimulation signal. In contrast to the IL-2 CD28RE, transcriptional activity of the IL-4 CD28REs can be activated by the CD28 signal alone. Upon CD28 stimulation, AP-1 (c-Jun, JunD) and NF-xB (c-Rel, p65/RelA) family proteins bind to...
these two elements and are involved in governing the CD28 responsiveness of the two elements. PRE-I and P1 belong to the most important regulatory elements of the IL-4 gene (26–28). Both sites are also shown to be involved in Th2-specific protein/DNA interactions and to be the potential regulatory elements for Th2-specific expression of the IL-4 gene (29, 30). The finding of PRE-I and P1 as CD28REs may lead to a better understanding of CD28-mediated activation of the IL-4 gene.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The cells used in this study were the human T-lymphoblastoid cell line Jurkat (J16-77). Cells were cultured in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum, 50 μg/ml gentamicin (Life Technologies), 6 mM HEPES (Life Technologies; 1 mM solution), and 2 mM l-glutamine (Life Technologies; 200 mM solution).

**RNA Isolation and Reverse Transcription-PCR Analysis of IL-4 mRNA**—Total cellular RNA was isolated using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. 1 μg of RNA was reverse transcribed with oligo(dT). PCR amplification was performed for 35 cycles with specific primers (Stratagene, Heidelberg, Germany) set at 960 microfarads, 240 V. The transfected cells were electroporated using a Bio-Rad Gene Pulser (Bio-Rad GmbH, München, Germany) set at 960 microfarads, 240 V. The transfected cells were further treated with α-CD3 mAb (OKT3 coated at 10 μg/ml), α-CD28 mAb (5 μg/ml), or α-CD3 plus anti-CD28 mAb for 8 and 16 h. Luciferase activity was determined in 10 μl of cell extract using the luciferase assay substrate (Promega) with an Duolumat LB9507 luminometer (Berthold, Germany).

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assays (EMSA)**—Nuclear extracts from Jurkat T cells unstimulated and stimulated for 2 h with either α-CD3 mAb (OKT3 coated at 10 μg/ml) or α-CD3 plus α-CD28 mAb (5 μg/ml) were prepared as described previously (29). EMSA was performed as published previously (29). Antibody analyses were done by preincubating the nuclear extracts with 1–2 μl of indicated antibodies for 20 min before adding the end-labeled probe. The synthetic oligonucleotides used for EMSA and competition experiments were as follows.

**Antibodies**—The α-c-Rel, α-c-Jun, α-c-JunD (329), α-c-Jun/AP-1 (broadly reactive with c-Jun, Jun B, and Jun p39 proteins), and α-c-Fos (k-25, broadly reactive with c-Fos, FosB, Fra-1, and Fra-2) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The α-NF-κB/RelA(p65) antibody was purchased from Rockland (Gilbertsville, PA). Antibody specific for c-Jun B was kindly supplied by Dr. P. Angel (German Cancer Research Center, Heidelberg, Germany). The α-NF-ATc mAb (MA3–024) was purchased from Affinity BioReagents, Inc. (Golden, CO).

**RESULTS**

Up-regulation of IL-4 Expression by CD28 Costimulation—CD28 costimulation has been shown to increase production of IL-4 protein both in human and mouse T cells (9, 12). To investigate the CD28 signal involved in expression of the human IL-4 gene at the transcriptional level, we chose an IL-4-
producing Jurkat T cell subline for our studies. Stimulation of Jurkat cells with phytohemagglutinin or α-CD3 (OKT3) mAb alone resulted in induction of IL-4 mRNA expression. The production of IL-4 mRNA was clearly increased upon co-stimulation with α-CD28 mAbs in Jurkat T cells (Fig. 1). CD28 is known to serve as a costimulatory cell surface molecule in T cell activation. Interestingly, we observed that stimulation of Jurkat cells with α-CD28 mAb alone could induce a low but significant level of IL-4 mRNA expression. The capability of α-CD28 mAb in induction of IL-4 mRNA expression was also observed in a second Jurkat T cell line (data not shown). This indicates that CD28 ligation does not only provide a costimulatory signal but may also directly induce expression of the IL-4 gene at the transcriptional level. The experiments also demonstrate that the CD28 signal is involved in generating a high level of IL-4 expression in Jurkat T cells.

To investigate the biological function of the human IL-4 promoter in response to CD3 and CD28 signals, the human IL-4 promoter fragment −269 to +11 (which contains the important regulatory elements identified so far) was placed in front of the luciferase reporter gene. Activities of the IL-4 promoter were analyzed by transfection of the IL-4-luciferase construct into Jurkat T cells. The data indicate that PRE-I and P1 are the binding sites responsive to CD28 signaling. For CD28-induced nuclear factors and, therefore, may be the sites responsible for CD28 signaling.

**Inducible Binding of Nuclear Factors to PRE-I and P1**—The IL-4 −269 to +11 promoter fragment contains multiple regulatory transcriptional elements (25). To study the CD28-responsive DNA binding sites of the IL-4 promoter, we prepared nuclear proteins from Jurkat T cells unstimulated and stimulated by α-CD3, α-CD28 or a combination of α-CD3 and α-CD28 mAbs and analyzed them by EMSA. Two regulatory elements, PRE-I at −250 to −221 (27) and P (also referred as P1) at −77 to −67 (26, 32) were shown to interact with nuclear proteins of cells stimulated with α-CD28 or α-CD3/α-CD28 mAbs (Fig. 3). Two complexes (PRE-I-I and PRE-I-II), detected by the PRE-I probe, and three complexes (P-I, P-I-II, and P-I-III) detected by the P1 probe were clearly shown in CD28-stimulated cells. Formation of P-I and P-I-1 was enhanced in cells stimulated with both α-CD3 and α-CD28 mAbs (Fig. 3). In contrast, PRE-I-I was not found in cells stimulated by α-CD3 mAb alone. Formation of PRE-I-I was not increased by CD28/CD3 costimulation. PRE-I-II and P-I-III could be detected in nonstimulated cells. Formation of these two complexes was increased upon induction. The data indicate that PRE-I and P1 are the binding sites for CD28-induced nuclear factors and, therefore, may be the sites responsive to CD28 signaling.

**PRE-I and P1 Confer CD28-dependent Induction**—To investigate the transcriptional activity of PRE-I and P1 upon CD28 costimulation, we constructed luciferase reporter plasmids each containing 1–3 copies of the PRE-I or the P1 element linked to a minimal promoter containing a TATA box only. The biological function of the two elements was analyzed by transfection of the constructs into Jurkat T cells. For controls, four copies of the NF-κB consensus binding site were also considered.
costimulated cells. In contrast to the cells stimulated by α-CD28 alone, in the α-CD3/α-CD28-stimulated cells, PRE-I-I was completely blocked by the α-c-Jun/AP-1 antibody (Fig. 6B), indicating that the components of PRE-I-I in α-CD3/α-CD28-stimulated cells may differ from those in α-CD28-stimulated cells. Indeed, α-JunB Ab, which did not interfere with the formation of PRE-I-I in the α-CD28-stimulated cells, reduced PRE-I-I in the α-CD3/α-CD28-stimulated cells (Fig. 6B). In contrast, α-c-Jun Ab interfered with the formation of PRE-I-I in α-CD28-stimulated cells but not in the α-CD3/α-CD28-stimulated cells. JunD was detected in the PRE-I binding complexes in both α-CD28- and α-CD3/α-CD28-stimulated cells. Fos was not found to participate in binding to PRE-I in α-CD28- or the α-CD3/α-CD28-stimulated cells.

Formation of PRE-I-II was enhanced after CD3 stimulation (Fig. 3). Competition experiments showed that formation of PRE-I-II was competed by NF-κB and NF-AT oligonucleotides (Fig. 5). Antibody analysis showed that PRE-I-II formed in α-CD3/α-CD28-treated cells, α-c-Jun/AP-1 antibody slightly supershifted PRE-I-I. No effect was obtained by the α-c-Fos antibody (Fig. 6A). This indicates that PRE-I-I may contain proteins of the Jun family. We further investigated the components of the PRE-I binding complexes using antibodies specifically against c-Jun, JunB, or JunD. Our experiments showed that PRE-I-I and PRE-I-II were supershifted by α-JunD Ab (Fig. 6A). Formation of PRE-I-I was also blocked by α-c-Jun Ab (Fig. 6A). The experiments demonstrate that CD28 stimulation leads to binding of c-Jun and JunD to PRE-I. We also investigated whether the components of PRE-I binding complexes changed in α-CD3/α-CD28-

![Fig. 5. Competition analysis of the PRE-I and P1 binding complexes.](http://www.jbc.org/)

The TATA-luciferase vector did not respond to any stimuli (Fig. 4). To characterize the α-CD3 and α-CD28 mAb-induced complexes, oligonucleotides containing binding sites for several known inducible factors were used as DNA competitors in EMSA. The experiment showed that formation of the CD28-inducible complex PRE-I-I was specifically competed by an excess of the unlabeled oligonucleotides containing the AP-1 and NF-AT binding sites (Fig. 5). To further investigate whether AP-1 proteins are involved in binding to PRE-I upon CD28 stimulation, α-c-Jun/AP-1, broadly reactive with c-Jun, JunB, and Jun p39 proteins and α-c-Fos (k-25), broadly reactive with c-Fos, FosB, Fra-1, and Fra-2, were used in EMSA. Antibody analysis showed that in α-CD28-treated cells, α-c-Jun/AP-1 antibody slightly supershifted PRE-I-I. No effect was obtained by the α-c-Fos antibody (Fig. 6A). This indicates that PRE-I-I may contain proteins of the Jun family. We further investigated the components of the P1 element.

![Fig. 4. The IL-4 PRE-I and P1 elements confer CD28-inducible transcriptional activity.](http://www.jbc.org/)

The TATA-luciferase vector did not respond to any stimuli (Fig. 4). To characterize the α-CD3 and α-CD28 mAb-induced complexes, oligonucleotides containing binding sites for several known inducible factors were used as DNA competitors in EMSA. The experiment showed that formation of the inducible P1 element was specifically competed by oligonucleotides containing the NF-AT and NF-κB consensus sequence (Fig. 5). The P1 probe used in our EMSA experiments did not contain the putative AP-1 site (25). Nevertheless, formation of all three inducible P1 com-
plexes was competed by the AP-1 oligonucleotide, indicating that AP-1 proteins might be involved in binding to the P1 site (Fig. 5). Antibody analysis of the P1 binding complexes of the α-CD28-stimulated cells showed that all three complexes were supershifted by the α-JunD Ab (Fig. 7). Therefore, JunD was also involved in binding to the P1 site in α-CD28-stimulated cells (Fig. 7A). Besides, α-p65/RelA and α-c-Rel Abs blocked formation of P1-I demonstrating that c-Rel and RelA of the NF-κB proteins were involved in binding to the P1 site upon CD28 stimulation (Fig. 7A). In α-c-CD3/α-CD28-stimulated cells, α-c-Fos and α-NF-ATc Abs also reduced formation of the P1 binding complexes, indicating that NF-AT and other members of the AP-1 family of proteins may form the P1-binding complexes upon CD3 stimulation (Fig. 7B). The above studies demonstrate that JunD, p65/RelA, and c-Rel are involved in binding to the IL-4 promoter regulatory elements during CD28 costimulation.

Engagement of the TCR leads to activation of two (the protein kinase C and the calmodulin-dependent) signal transduction pathways. It is known that PMA activates protein kinase C and that calcium ionophores (such as ionomycin and A23187) increase the level of intracellular free Ca$^{2+}$. Therefore, the physiological stimulation of T cells via TCR may be mimicked by the combination of PMA and ionomycin. Previously, Casolaro et al. (34) reported that activation of human IL-4 transcription through A23187 was diminished by PMA in Jurkat T cells and that overexpression of RelA down-regulated human IL-4 promoter activity in A23187-stimulated Jurkat T cells. They proposed that NF-κB (activated by PMA) may compete with NF-AT (activated by A23187) for binding to the P1 site and, consequently, inhibit NF-AT-dependent transcription at this site. To investigate the functional relevance of NF-κB on the P1 site upon CD3 stimulation, we cotransfected the P1 multimer construct together with a RelA or a c-Rel expression plasmid into Jurkat T cells. As shown in Fig. 8, overexpression of RelA or c-Rel increased the P1-mediated transcription in CD3-stimulated Jurkat T cells. Therefore, NF-κB may enhance transcriptional activity at the P1 site in physiologically stimulated T cells.

DISCUSSION

In recent years, experimental evidence showed that signal transduction through CD28 not only augmented TCR-mediated proliferation, IL-2 production, and T cell survival but also played an important role in differentiation of Th cells into either type 1 or type 2 cells (11, 35). CD28 costimulation has been shown to promote production of Th2 cytokines including IL-4 (12). Blocking the CD28-B7 interaction blocks the induction of IL-4 synthesis (5, 6, 36), indicating that CD28 costimulation is important in regulation of IL-4 synthesis. Since IL-4 is not only a critical cytokine for Th2 differentiation but also plays a central role in the pathogenesis of allergic inflammation, we investigated the CD28-responsive element of the human IL-4 promoter. We show here that IL-4 mRNA levels and activity of the IL-4 promoter are increased by CD28 costimulation of α-CD3- or mitogen-activated Jurkat T cells. Interestingly, in addition, we found that α-CD28 mAb alone could induce IL-4 mRNA expression and activate the IL-4 promoter to a significant level. This indicates that CD28 ligation, so far known to provide a costimulatory signal in T cell activation, may also directly activate transcription. Two of the IL-4 promoter regulatory elements, PRE-I and P1, are shown to confer CD28-stimulation-induced transactivation.

PRE-I was previously shown to interact with AP-1 and NF-AT in Th2 cells (29). AP-1 transcriptional activity in T cells has been shown to be strongly induced by α-CD28 induction (37), and CD28 costimulation is necessary for the optimal activation of AP-1, via c-Jun phosphorylation (33). Here, we show that the multimerized PRE-I element may contribute to an approximately 4-fold transcriptional activation in response to CD28 signaling. Our experiments also show that binding of c-Jun and JunD to PRE-I is specifically induced via CD28 stimulation. These data indicate that PRE-I may be a CD28-responsive element in the IL-4 gene.

P1 contains a strong NF-AT binding site (24, 25). It also shares DNA sequence similarity with the DNA binding sequence for NF-κB and binds both NF-AT and proteins of the NF-κB family (34). The corresponding P1 sequence in the human IL-4 promoter differs by two nucleotides from the mouse sequence (TGGAAAAATTTT instead of TGGAAAAATTTT) and was shown to bind NF-AT with 4-fold lower affinity and NF-κB with 4-fold higher affinity than does the mouse sequence (34). It has been well established that the Rel-related κB proteins are downstream targets in CD28 signaling (38–42). CD28 stimulation can directly activate the NF-κB/Rel family of transcription factors via down-regulating the expression of the NF-κB inhibitor I-κB and increasing the nuclear translocation of NF-κB/Rel (22, 24, 43, 44). NF-κB/Rel transcription factors are found to participate in the formation of the IL-2 CD28-responsive element-specific complex and to be involved in CD28-stimulated transactivation (21–23). We show here that JunD,
c-Rel, and p65/Rel are involved in binding to the IL-4 promoter P1 element after CD28 stimulation. The multimerized P1 element confers 6–8-fold inducible transcriptional activity to the reporter gene upon CD28 stimulation. The data suggest that P1 may be the second CD28-responsive site of the IL-4 gene and that JunD and Rel-related proteins are involved in activation at this site.

PRE-I and P1 elements were previously shown to be essential for expression of the human (27) and murine (28) IL-4 gene. Deletion or mutation of PRE-I and P1 leads to impairment of both basal and inducible activity of the IL-4 promoter (26–28, 31). Recent studies indicate that PRE-I and P1 may play an important role in the differential regulation of IL-4 gene expression levels in Th2 cells (29, 30). The finding of PRE-I and P1 as the CD28-responsive sites further confirms the importance of the two elements for IL-4 gene expression and also demonstrates that the CD28 signal regulates IL-4 gene expression through the most important regulatory elements.

Differences between the IL-2 CD28RE and the IL-4 PRE-I and P1 are observed. 1) Inducible binding of nuclear factors to the IL-2 CD28RE requires both the CD28-induced signal together with either phorbol myristate acetate or α-CD3 (19). Unlike the IL-2 CD28RE, inducible binding of nuclear proteins to the IL-4 PRE-I and P1 elements is readily detected in nuclear extracts prepared from α-CD28-stimulated cells. 2) The CD28 signal alone cannot activate the IL-2 CD28RE (23). In contrast, IL-4 PRE-I and P1 elements may function as a “pure” CD28RE and respond to CD28 stimulation alone. 3) Although NF-κB/Rel family members make up the IL-2 CD28RE protein complex, the IL-2 CD28RE does not function independently but works only in conjunction with the adjacent proximal AP-1 binding site (20). We show here that the IL-4 P1 element binds both NF-κB/Rel and AP-1 family members, although the P1 probe used does not contain an AP-1 site. This indicates that NF-κB and AP-1 proteins may interact with each other to bind together to a NF-κB-like regulatory element upon CD28 stimulation.

Using reagents such as PMA and A23187 for T cell stimulation, Casolaro et al. (34) found that activation of human IL-4 transcription through A23187 (the Ca²⁺-dependent pathway) was diminished by PMA (protein kinase C stimulation). They proposed that NF-κB (activated by PMA) may compete with NF-AT (activated by A23187) for binding to the P1 site and, consequently, inhibit the NF-AT-dependent transcription at this site. In our experiments, we stimulated Jurkat T cells through TCR by α-CD3 mAb coated at 10 μg/ml. Under these conditions, the activity of the multimerized P1 element was augmented by CD28 costimulation. Overexpression of RelA/p65 and c-Rel increased the P1-mediated transcription in CD3-stimulated Jurkat T cells. Therefore, NF-κB can activate transcription at the P1 site at physiological conditions of stimulation. However, when Jurkat cells were optimally activated by α-CD3, the P1 multimer reporter activity (but not the IL-4 promoter activity) was reduced by 10–15% when costimulated with α-CD28 mAb (data not shown). Therefore, as reported by Casolaro et al. (34), NF-κB may exert a negative effect at the P1 site when transcription is maximally regulated by NF-AT. Even in this case, the IL-4 promoter activity was augmented upon CD28 costimulation indicates positive involvement of other CD28-responsive elements (such as PRE-I).

Transcriptional regulation of the IL-4 gene is controlled by multiple regulatory promoter elements. Therefore, action of other, yet undefined DNA-binding factors may also be involved in the transcriptional synergy observed upon CD28 costimu-
tion. Besides PR-1 and P1, the IL-4 promoter also contains several composite sites that bind AP-1 and NF-AT cooperatively (31, 45). Activation of T cells by α-CD28 has been shown to strongly induce AP-1 transcriptional activity via posttranslational modification (37). Therefore, sites involved in interaction with AP-1 may also contribute to transcriptional activation in response to CD28 signaling.

Stimulation of the CD28 surface molecule has also been shown to specifically regulate the stability of messenger RNAs for IL-2, interferon-γ, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor (46). Thus, increase of RNA stability via the CD28 pathway may be one of the mechanisms to account for the CD28 costimulation-enhanced gene expression. Whether this mechanism is also involved in the synergistic effects of CD28 costimulation on the expression of the IL-4 gene needs to be investigated.

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