Tumor Necrosis Factor-α Activation of NF-κB Requires the Phosphorylation of Ser-471 in the Transactivation Domain of c-Rel*

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Activation of the transcription factor NF-κB is controlled at two levels in resting T cells: an initial activation induced by the triggering of the TcR-CD3 complex and a second phase controlled by paracrine- or autocrine-secreted TNFα. The initial phase is regulated by p65 (RelA), whereas the second one is mainly dependent on c-Rel. We describe here a mutant clone, D6, derived from the parental T lymphoblastic line Jurkat that fails to activate NF-κB upon TNFα stimulation. This clone had no alteration in tumor necrosis factor-α (TNFα) signaling pathways nor in IκBα, -β, or -ε expression and degradation. However, TNFα induced an exacerbated apoptotic response in this clone compared with Jurkat cells. This mutant clone showed a defect in the intermediate-late translocation of c-Rel to the nucleus promoted by TNFα stimulation, whereas early translocation is not affected. Activation or translocation of p65-containing complexes was not altered in this mutant clone. Sequencing of the c-Rel gene from this clone revealed a mutation of Ser-471 to Asn in the transactivation domain. The mutant S471N transactivation domain fused to the Gal4 DNA binding domain could not be activated by TNFα, unlike the wild type. Moreover, the overexpression of the mutant protein c-Rel S471N into Jurkat cells abolished TNFα-induced NF-κB activity, thus demonstrating that this mutation is responsible for the failure of TNFα stimulation of NF-κB. Moreover, extracts from TNFα-stimulated Jurkat cells phosphorylated in vitro recombinant wild type GST-c-Rel 464–481 but not the GST-c-Rel mutant. Thus, TNFα-induced phosphorylation of Ser-471 seems to be absolutely necessary for TNFα activation of c-Rel.

Activation of T cells is a biphasic process (1). Early events following TcR-CD3 ligation trigger the expression of several genes (such as the IL-21 and IL-2 receptor α chain) or tumor necrosis factor-α (TNFα) genes that are essential regulators of the subsequent second phase of T cell proliferation and acquisition of effector functions (2–4). This program of gene expression is controlled by the regulated activation of several transcription factors such as NF-AT, AP-1, and Oct-1 (2, 3). Among the most important factors involved in this process of T cell activation are those belonging to the NF-κB family, which regulates several of the most important genes induced during T cell activation (for review see Ref. 5). NF-κB is rapidly activated by the TcR-CD3 complex, but at later phases of T cell activation, autocrine- or paracrine-secreted TNFα takes control of NF-κB activation. The initial phase of NF-κB activation depends on p65 translocation, whereas the later TNFα-dependent phase is controlled by c-Rel (1).

The NF-κB family of transcription factors is composed of homo- and heterodimers of a family of proteins that includes the Dorsal gene of Drosophila and the mammalian genes nfkb1, nfkb2, c-rel, relA (p65), and relB (for review see Ref. 6). All members share a conserved 300-amino acid region in their N-terminal portion that includes the dimerization, nuclear localization, and DNA binding regions. c-Rel, RelB, and RelA also have C-terminal transactivation domains that strongly activate transcription from NF-κB sites. NF-κB is regulated at least in part by its subcellular localization. Thus, functional NF-κB complexes are held in the cytoplasm of resting T cells in an inactive state complexed with members of the IκB family. In response to different activators (which include TcR-CD3 and TNFα), IκB is phosphorylated by IκB kinases and subsequently degraded, liberating the active NF-κB complex, which translocates to the nucleus and activates transcription (for review see Ref. 7).

Recently a second level of regulation of NF-κB activation that involves the direct activation of transcriptional-competent NF-κB family members has been emerging. Thus, the catalytic subunit of protein kinase A was shown to be bound to inactive NF-κB complexes, and upon IκB degradation, this catalytic subunit phosphorylated p65, resulting in an enhanced transcription-promoting activity (8). Moreover, it was reported that TNFα treatment of cells resulted in phosphorylation of Ser-529 in the transactivation domain of p65, resulting in the activation of the transcriptional activity of the protein (9). The small GTP-binding protein Ras enhanced p65/RelA transcriptional activity through a pathway that required the stress-activated protein kinase p38 or a related kinase (10), although it was not demonstrated whether this kinase was directly involved in activating NF-κB or a transcriptional co-activator. The activity of Ras as well as the atypical protein kinase Cζ has been also shown to be essential for the transcriptional activity of p65/RelA in endothelial cells (11). This activation relies in the phosphorylation of the N-terminal Rel homology domain and not on the C-terminal transactivation domain. Other downstream effectors of Ras such as Raf/mitogen-activated protein kinase kinase (MEK), other small GTPases, phosphatidylinositol 3-kinase (PI3K), and the stress-activated protein kinase pathway were not involved in this Ras and protein kinase Cζ-dependent Rel homology domain p65 phosphorylation. On the other hand, the IL-1-induced NF-κB activity has been recently found to be dependent on PI3K activity (12). The acti-
vation of PI3K triggers a signaling cascade that leads to the specific phosphorylation of p65/RelA subunit. This phosphorylation enhances p65-mediated transcription without affecting IxB degradation, nuclear translocation of NF-xB, or the ability of NF-xB to bind to DNA. Nevertheless, the specific site(s) of phosphorylation was not identified.

In this work we report that a mutation in Ser-471 to Asn within the transactivation domain of c-Rel results in the abrogation of TNF-a-induced NF-xB activity in T cells. This indicates the existence of a second level of regulation of c-Rel activity by TNF-a through modulation of the activity of the transactivation domain.

**EXPERIMENTAL PROCEDURES**

**Cells—**Jurkat cells were grown in Dulbecco’s modified essential medium (Life Technologies, Inc.) supplemented with 5% heat-inactivated fetal bovine serum (Life Technologies, Inc.) and containing 100 µg/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine, plus nonessential amino acids at 37 °C in 7% CO2. D6 Jurkat cells were subjected to autoradiography. 

**Antibodies—**Sera from rabbits hyperimmunized with peptides derived from the c-Rel (aa 225–235 [1]226), (aa 925–935 [1]227), and IxB (aa 1775) [1]228, kindly provided by Dr. Nancy Rice (NCI-Frederick Cancer Research and Development Center, Frederick, MD), were used to detect the corresponding proteins on Western blots. All of them were used at a dilution of 1:1,000 except serum #265, which was at a 1:10,000 dilution. Polyclonal antibodies against Jun kinase (JNK) (sc-474; Santa Cruz, CA) and β-adrenergic receptor kinase (kindly provided by Dr. Federico Mayor, Centro de Biología Molecular Severo Ochoa, Madrid) were used for immunoprecipitation before immunocomplex kinase assays.

**Reagents—**Recombinant human TNF-a was purchased from Genzyme (Cambridge, MA). Phorbol myristate acetate (PMA) and calcium ionophore A23187 were purchased from Sigma. Cycloheximide was purchased from Roche Molecular Biochemicals. Casein was purchased from Sigma.

**Plasmids—**The pNF3TTLuc reporter plasmid contains a trimer of the NF-xB binding motif of the H-2k gene upstream of the thymidine kinase minimal promoter and the luciferase reporter gene (13). The pNF3ConA luc reporter contains three tandem repeats of the human immunodeficiency virus-1 long terminal repeat NF-xB enhancer upstream of the thymidine kinase promoter and the luciferase reporter gene (kindly provided by Dr. J. Alcamí, Hospital 12 de Octubre, Madrid). Gal4-c-Rel transactivation domain 309–588 wild type and 309–588 S471N were made by cloning the corresponding PCR fragments into the Xhol-BglII site of the Gal4-c-Jun 1–166 plasmid, thus removing the c-Jun fragment. The templates for PCR reactions were pRC-hc-Rel (which consists of pRT-CMV with c-Rel cDNA inserted in the HindIII-Xbal site) and pRC-Rel/S471N (site-directed mutagenesis of c-Rel nucleotide 1685 from G to A was accomplished using the MutaGene phagemid mutagenesis kit (Bio-Rad) with pRC-hc-Rel as template).

**Apoptosis Measurement—**Jurkat or D6 cells (5 x 106 cells) were stimulated with or without TNF-a (10 ng/ml) in the presence of 1 µg/ml cycloheximide for 24 h before washing twice with ice-cold phosphate-buffered saline. Cells were then incubated with buffer staining 0.1% sodium citrate, 0.3% Nonidet P-40 and 0.05% propidium iodide (Sigma), and cell cycle analysis was immediately performed in a FACScalibur (Becton Dickinson, San Jose, CA) flow cytometer. Apoptosis was recorded as the percentage of cells with haplo-diploid content of DNA.

**Electrophoretic Mobility Shift Assay—**Preparation of nuclear extracts was described in detail elsewhere (14). The binding reaction consisted of 5 µg of extracted nuclear protein and 5 µg of poly(dI-dC) (Roche Molecular Biochemicals) in a reaction volume of 10 µl containing 6 mM MgCl2. This mixture was then incubated at room temperature for 10 min, after which 2 µl (50,000 cpm) of the NF-xB consensus oligonucleotide (Promega, Madison, WI) end-labeled with [32P]ATP (specific activity = 3,000 Ci/mmol; Amersham Pharmacia Biotech), was added. A control reaction mixture was always included in which a 100× molar excess of unlabeled reference NF-xB oligonucleotide was added to verify the specificity of the binding reaction. After incubation in an ice bath for 15 min, the reaction mixtures were run on 5% PAGE. After drying, the gels were subjected to autoradiography.

**Western Blots—**Whole cell extracts (WCE) were made using TNT buffer as the lysis buffer (20 mM Tris-HCl, pH 7.6, 200 mM NaCl, 1% Triton X-100) supplemented with protease inhibitors (2 µg/ml aproteminin, 2 µg/ml pepstatin, 2 µg/ml leupeptin, 0.1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (5 mM NaF, 100 µM sodium orthovanadate). Nuclear extracts were prepared as described in the preceding section. 10% of whole cell extracts or 5 µg of nuclear extracts were run on a 10% SDS-PAGE and transferred to a polycrylindene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Rabbit anti-human p65, c-Rel, IxBa, IxBj, or IxBε, as appropriate, was used as the first antibody, and goat anti-rabbit IgG peroxidase was used as the secondary antibody. The enhanced chemiluminescent (ECL) developing kit (Amersham Pharmacia Biotech) was used to identify the relevant band(s).

WCE made from 106 cells were immunoprecipitated in TNT buffer with 1 µl of serum. Precipitates were collected on protein A-Sepharose (Amersham Pharmacia Biotech) and separated in a 10% SDS-PAGE and subsequently transferred to a polycrylindene difluoride membrane. Membranes were analyzed by Western blot.

**cDNA and Genomic DNA Sequencing—**Total RNA from 106 Jurkat or D6 cells was extracted using Trizol (Life Technologies, Inc.) following the manufacturer’s directions. 1 µg of each sample was used for cDNA synthesis using the GeneAmp RNA PCR core kit (Perkin-Elmer) following kit instructions. The 5′ sense primer for c-Rel cDNA amplification was CTCTGACTGACTGACTGACTG, and the corresponding 3′ sense primer was ATGGCTCAAGTTTGATG. Both primers were also used for sequencing in an automatic Perkin-Elmer sequencer. For PCR amplification of c-Rel exon VII (comprising the transactivation domain), the 3′ antisense primer was the same as above, whereas the 5′ sense primer was CACTTCTCTTCAATTCG. The latter primer was also used for sequencing.

**Transfection and Stimulation of Jurkat T Cells—**Jurkat cells were washed once and resuspended at 105 cells/ml in OPTI-MEM medium (Life Technologies, Inc.). Cells were transfected with the LipofectAMINE Plus reagent (Life Technologies, Inc.) in the preparation of the LipofectAMINE Plus-plasmid mixtures, in accordance with the manufacturer’s instructions. The mixtures were incubated at 37 °C in a 7% CO2 incubator for 3 h before washing with fresh Dulbecco’s modified essential medium plus 5% fetal bovine serum and incubated further for 18 h. The cells were then washed once with, and resuspended at the same concentration in, Dulbecco’s modified essential medium plus 5% fetal bovine serum. Cultures were incubated with or without 10 ng/ml of TNF-a or PMA (10 ng/ml) plus calcium ionophore (1 µg/ml) was added to duplicate wells containing 0.5 ml of these cell suspensions, which were then incubated under the same conditions for 6 h. The cells were lysed with passive cell culture lysis reagent (Promega), microcentrifuged at full speed for 5 min at 4 °C, and 20 µl of each supernatant was used for luciferase supernatant activity. The results were expressed as increases in luminescence relative to the value obtained with the non-stimulated control after normalization with respect to protein concentration determined by the bicinchoninic acid spectrophotometric method (Pierce).

**Solid-phase in Vitro Phosphorylation Assay—**c-Rel transactivation domain (residues 464-481 (using as template pRc-hc-Rel wild type or pRc-hc-Rel S471N) was cloned into the BamHI-EcoRI site of plasmid pGEX2T (Amersham Pharmacia Biotech) to express recombinant GST-c-Rel fusion protein. These recombinant proteins were purified from Escherichia coli-induced cultures according to the manufacturer’s instructions. 25 µl of GSH-agarose-GST-c-Rel were used as substrate of in vitro phosphorylation reaction in which whole cell extracts from non-stimulated or stimulated Jurkat cells were assayed. WCE were made from 106 Jurkat cells in 25 µl, as described previously. The reaction mixture contained 20 µl Hepes, pH 7.6, 20 mM MgCl2, 20 mM β-glycerophosphate, 20 µM ATP, and 1 µCi of [32P]ATP (specific activity 3,000 Ci/mmol). After 20 min at 30 °C, the reaction was terminated by adding with TNT buffer. Phosphorylated protein was boiled in 20 µl of Laemmli sample buffer and resolved in 10% SDS-PAGE, followed by autoradiography. For JNK and β-adrenergic receptor kinase activity assays, the corresponding kinase was immunoprecipitated as described from WCE, and the immunoprecipitate was subjected to an in vitro phosphorylation assay using GST-c-Jun (1–147) fusion protein and casein as substrates, respectively.

**Orthophosphate Labeling—**Jurkat cells were incubated with phosphate-free medium for 16 h before 3 mCi of [32P]orthophosphate per million cells were added. After 6 h of incubation, replicate plates were left untreated or stimulated with 10 ng/ml TNF-a for 15 min. After that period of time, WCE were made as described, and resulting extracts were immunoprecipitated with anti-c-Rel antisera 265, as described previously. Immunoprecipitates were resolved in a 10% SDS-PAGE and developed by autoradiography.
to TNFα. We were able to identify one clone, named D6, that had very little, if any, NF-κB reporter inducibility when stimulated with TNFα (Fig. 1A). This failure of TNFα responsiveness was not due to deficient expression of TNF receptors, since clone D6 expressed the TNF receptors at levels comparable with Jurkat cells (data not shown). Moreover, stimulation with a potent T cell mitogenic stimulus, such as PMA plus calcium ionophore, led to strong NF-κB reporter activity in clone D6 cells (Fig. 1A), indicating the specificity of the defect to TNFα stimulation.

We tested whether this lack of responsiveness to TNFα was specific for NF-κB activation or whether it also extended to other TNFα-mediated responses. The D6 clone showed an enhanced apoptotic response to TNFα, as compared with parental Jurkat cells (Fig. 1C), indirectly indicative of a specific failure of TNFα to activate NF-κB. We next tested whether this lack of responsiveness was due to a defect in the TNFα signaling mechanisms, not only in the NF-κB pathway. Since AP-1 is another transcription factor activated by TNFα, we tested the activity of JNK using in vitro phosphorylation assays with GST-c-Jun as a substrate. TNFα activated JNK activity in both Jurkat and clone D6 cells. As a control we immunoprecipitated β-adrenergic receptor kinase (GRK2) from the same extracts and tested its kinase activity using casein as substrate. We used β-adrenergic receptor kinase (GRK2) as a control because this kinase is highly expressed in T cells, but it is activated by G-protein-coupled receptors, not by TNFα receptors (15). As expected, GRK2 activity was not induced by TNFα, whereas both Jurkat and clone D6 cells showed a similar basal activity (Fig. 1B). Therefore, although the TNFα induction of NF-κB was abnormal in clone D6, other TNFα-mediated pathways appeared normal.

Next, we tested whether this lack of activity resulted from the inability of TNFα to promote nuclear translocation of NF-κB. As shown in Fig. 2A, gel-shift assays with a NF-κB consensus oligonucleotide using nuclear extracts from TNFα-treated Jurkat cells showed the presence of two NF-κB binding complexes, labeled I and II, peaking from 30 min to 4 h and still present at 15 h. Surprisingly, there was also induction of NF-κB binding complexes in clone D6 cells by TNFα stimulation, although this induction was less sustained over time than that observed in Jurkat cells, peaking at 30 min but then returning progressively to basal levels (Fig. 2A). Furthermore, the kinetics of nuclear translocation of NF-κB promoted by PMA plus ionophore treatment was similar in both Jurkat and clone D6 cells (Fig. 2B). To analyze the composition of the two complexes I and II, we used supershift assays where nuclear extracts were treated with antibodies directed against different NF-κB family members before the addition of the probe. Both complexes I and II disappeared when the extracts were treated with anti-p65 antibody and also when anti-p65 and anti-c-Rel were used together (data not shown), indicating that these complexes were made from heterodimers of p50/p65 and p50/c-Rel. Incubation with anti-p65 antibody alone revealed the presence of unshifted c-Rel-containing complexes (Fig. 2C, lanes 2, 5, 8, and 11). Conversely, incubation with anti-c-Rel antibody revealed the presence of unshifted p65 complexes (Fig. 2C, lanes 3, 6, 9, and 12). Thus, complex I contained p50/p65 heterodimers, whereas complex II was composed of heterodimers of c-Rel/p50. Interestingly, this experiment showed a difference in the relative composition of NF-κB complexes between Jurkat and clone D6. In control Jurkat cells, the relative amount of c-Rel in the DNA binding complexes (complex II) increased with time of TNFα treatment (Fig. 2C, compare the unshifted band in lanes 2 and 5). However, this increase was not seen in D6 cells (Fig. 2C, lanes 8 and 11).
We then directly analyzed the presence of c-Rel and p65 proteins in nuclear extracts from TNFα-stimulated cells by Western blot. We found that in both Jurkat and clone D6 cells, TNFα induced translocation of p65 with the same kinetics (Fig. 3A), maximum translocation was observed at 30 min, with return to basal levels at 4 h. c-Rel was also translocated to the nucleus at 30 min but reached a maximum at 4 h and maintained this plateau at least for 15 h after stimulation in wild-type Jurkat cells. In clone D6 cells, this translocation also started at 30 min, but it was not sustained in time, returning to basal levels by 4 h. These results are indicative of a failure in nuclear translocation of NF-κB complexes but not the inhibition of NF-κB-dependent reporter activity.

**IkB Degradation Is Not Affected in the Jurkat D6 Clone**—The previous results indicated a defect in c-Rel activation by TNFα in clone D6. One possibility was a differential association of c-Rel NF-κB complexes with different members of the IκB family, in such a way that c-Rel complexes in clone D6 cells could not be activated by TNFα as they were in wild-type Jurkat cells. To test this hypothesis, we immunoprecipitated IκBa, IκBβ, and IκBe from non-stimulated Jurkat and clone D6 cells and blotted with anti-p65 and anti-c-Rel antibodies to assess the composition of the complexes bound to IκBa, IκBβ, and IκBe, respectively. There were no significant differences between Jurkat cells and clone D6. Both IκBβ and IκBe were equally bound to both c-Rel and p65, while IκBa was bound predominantly to p65 (Fig. 4A). Since these experiments did not rule out the possibility that the kinetics of IκB degradation were different, we stimulated Jurkat and clone D6 cells with TNFα for up to 120 min in the presence of cycloheximide to analyze the degradation of IκB proteins (Fig. 4B). IκBa was degraded after 30 min of TNFα stimulation in both Jurkat and clone D6 cells, whereas IκBβ and IκBe were not affected at all. Once again, there were no significant differences between clone D6 and Jurkat with respect to IκB degradation kinetics upon TNFα stimulation. Indirectly, those results also indicate that no significant differences in the amount of c-Rel, p65, or IκB proteins exist between both cell types. This has been confirmed by Western blots of cytoplasmic extracts (data not shown).

Thus, the mechanisms leading to IκB degradation remained unaltered in clone D6, explaining the observed TNFα-induced translocation of NF-κB complexes but not the inhibition of NF-κB-dependent reporter activity.

**D6 Cells Expressed a Mutated Form of c-Rel**—Another possibility that could account for the defect in c-Rel activation by TNFα in clone D6 cells was a defect in c-Rel itself. To test this hypothesis we sequenced the entire c-Rel gene in Jurkat and clone D6. We isolated total RNA from both cell types and used it as template for a reverse transcription-PCR reaction. Primers specific for c-Rel were used to amplify c-Rel cDNA, and the same primers were used for sequencing. Interestingly, we found a point mutation at position 1685 (G to A change) in c-Rel cDNA obtained from D6 cells. This resulted in a change of
and are regulated independently of IκBα and IκBβ, and are directed reporter activity. This system has the advantage that the region of interest (c-Rel amino acids 309 to 588) downstream of the Gal4 DNA binding domain (Gal4-DBD), transfected the construct into Jurkat cells, and assayed for Gal4 directed reporter activity. This system has the advantage that the Gal4-transactivator fusion proteins are exclusively nuclear and are regulated independently of IκBα. Both wild type and S471N fusions showed substantial basal reporter activity by themselves, without any exogenous stimuli, whereas Gal4 DBD or Gal4 DBD c-Rel 309–318 (which comprises only the first 10 amino acids of c-Rel transactivation domain) were inactive (Fig. 5B). However, only the wild type but not the S471N mutant further activated transcription of the reporter when the transfected cells were stimulated with TNFα (Fig. 5C). In contrast, PMA + ionophore stimulated both the S471N mutant construct as well as the wild type. These results perfectly correlate with the defect observed in clone D6 upon TNFα stimulation using NF-κB luciferase reporter assays and suggested that this defect was due to the mutant c-Rel.

To further corroborate this, we transfected the Gal4 c-Rel 309–588 wild type and mutant S471N into clone D6 and assayed for TNFα or PMA plus ionophore stimulation of reporter activity. Both wild-type and mutant constructs induced a similar basal reporter activity, as in Jurkat wild-type cells. Interestingly, TNFα stimulation in clone D6 cells transfected with the wild-type construction was similar as the induction obtained in Jurkat cells, whereas Jurkat wild-type cells transfected with S471N mutant construction showed no induction upon TNFα stimulation (Fig. 5D). This result clearly indicated that the signaling mechanisms involved in c-Rel activation by TNFα were unaffected in clone D6, pointing at Ser-471 as a critical residue in c-Rel, responsible for TNFα activation. As expected, PMA plus ionophore stimulated both the wild-type and the S471N mutant constructions.

**Overexpression of S471N Mutant Inhibited TNFα Stimulation of NF-κB Activity**—Since mutant S471N transactivation domain was not activated by TNFα stimulation, we tested whether this mutation was responsible for the phenotype observed in D6 cells. For this, we co-transfected c-Rel mutant S471N into wild-type Jurkat cells along with a NF-κB luciferase reporter plasmid and assayed the activity induced by TNFα stimulation. As observed in Fig. 6, NF-κB basal activity was not significantly altered by both mutant or wild-type overexpression. Interestingly, TNFα stimulation did not further increase NF-κB activity in wild-type cells transfected with c-Rel mutant S471N, in contrast to empty vector transfected cells. Therefore, overexpression of c-Rel mutant S471N could reproduce the phenotype observed in D6 cells. Moreover, overexpression of wild-type c-Rel in D6 cells could significantly, although incompletely, increase TNFα-induced NF-κB activity in D6 cells, thus partially recovering the wild-type phenotype (Fig. 6). The lack of complete recovery could indicate that endogenous S471N protein was acting as a dominant negative mutant.

**TNFα-induced Phosphorylation of c-Rel Transactivation Domain**—As previous work on v-Rel and avian c-Rel (5, 8, 9) showed that c-Rel is phosphorylated, we hypothesized that a phosphorylation event in Ser-471 was implicated in c-Rel activation by TNFα. Metabolic labeling of Jurkat cells with [32P]orthophosphate showed that c-Rel is a phosphoprotein and that its phosphorylation was increased by TNFα treatment (Fig. 7A). To test if the induced phosphorylation produced by TNFα specifically takes place in the c-Rel transactivation domain, we used GST-c-Rel 421–588 (which includes all the c-Rel transactivation sequences) wild type and mutant S471N fusion proteins as substrates for a solid-phase kinase phosphorylation assay. Whole cell extracts from Jurkat cells and clone D6 cells stimulated or not with TNFα or PMA were used for this phosphorylation assay. Both the wild type and mutant S471N c-Rel fusion proteins were strongly phosphorylated by one or more kinase activities present in unstimulated Jurkat cells. This phosphorylation was further increased in extracts from TNFα or PMA plus ionophore-treated cells in both wild type and mutant S471N (data not shown), demonstrating that TNFα (as well as PMA plus ionophore) stimulation promotes the phosphorylation of c-Rel.

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transactivation domain. This suggested that Ser-471 would not be the only residue that is phosphorylated upon TNFα activation, although functional data reveal its critical role in TNFα activation of c-Rel. Thus, we then used a smaller construct comprising residues 464 to 481 to eliminate the background produced by other activation-dependent sites. The GST-c-Rel 464–481 recombinant protein was used as a substrate for a similar solid phase in vitro phosphorylation assay. As shown in Fig. 7B, the recombinant wild-type protein was slightly phosphorylated by the extract from unstimulated cells, and this phosphorylation was strongly increased by extracts obtained after TNFα or PMA plus ionophore stimulation. In contrast, phosphorylation in the mutant Ser-471 c-Rel was not increased upon stimulation of cells, indicating that the Ser at position 471 is involved in the specific phosphorylation of c-Rel in a TNFα-dependent manner.

**DISCUSSION**

NF-κB activation in resting human T cells upon TcR activation has been shown to be controlled in a biphasic manner (1). Thus, nuclear translocation of p65/p50 and c-Rel/p50 heterodimers triggered by the TcR-CD3 complex stimulation was observed at early times, whereas at intermediate-late times the active NF-κB complexes are mainly composed of c-Rel/p50 heterodimers. Interestingly, those intermediate-late events are controlled by autocrine or paracrine secretion of TNFα (1). Therefore, c-Rel emerges as the main NF-κB family member stimulated by TNFα in the context of physiologic activation of resting T cells. This biphasic model of NF-κB activation is in perfect agreement with the results found in the c-Rel knock-out mouse, which are deficient in primary T cell activation (17), (18). Those c-Rel null lymphocytes cannot proliferate to mitogenic stimuli, but they can be rescued by cytokines such as IL-2 to acquire the effector phenotype, thus demonstrating that c-Rel regulates the late phase of T cell activation. We report here the requirement of c-Rel for sustained NF-κB activity...
**c-Rel Ser-471 Is Critical for TNFα Activation**

**FIG. 7.** TNFα-induced phosphorylation of c-Rel transactivation domain. A, TNFα-induced phosphorylation of c-Rel in vivo. Jurkat cell cultures were labeled with [32P]orthophosphate and stimulated or not with TNFα for 15 min. WCE from those cultures were immunoprecipitated with anti-c-Rel antiserum and electrophoresed in a 10% SDS-PAGE. B, TNFα induced a kinase activity able to phosphorylate c-Rel Ser-471. Recombinant GST-c-Rel 464–481 wild type (lanes 1–3) and S471N mutant (lanes 4–6) proteins were used as substrates for in vitro solid-phase phosphorylation assays using whole cell extracts from non-stimulated cells or cells stimulated with TNFα or PMA plus ionophore (fo) for 30 min, as indicated. As controls, GST-c-Rel transactivation domain proteins were incubated in the absence of cellular extract, and GST alone was incubated with extract from TNFα-stimulated cells; in both cases, the result was no band (not shown). The intensity of the bands was determined by densitometry of the film, and the optical density (O.D.) units are plotted.

A

![Diagram showing TNFα phosphorylation of c-Rel](image)

B

|            | GSTc-Rel wt | GSTc-Rel S471N |
|------------|-------------|----------------|
| TNFα       | - +         | - +            |
| PMA+Ionophore | - +       | - +            |

![Graph showing phosphorylation levels](image)

Description:

- **A** shows the phosphorylation of c-Rel by TNFα.
- **B** shows a graph comparing TNFα and PMA+Ionophore stimulation levels, with control, TNFα, PMA, and PMA+Ionophore conditions.

After TNFα stimulation, we found a mutant Jurkat T cell clone, termed clone D6, that is defective in stimulated NF-κB-dependent promoter activity when cells were activated by TNFα but not by PMA plus ionophore (Fig. 1A). This defect, however, does not involve down-regulation of TNF receptors or intracellular signaling, since it shows a normal JNK activation in response to TNFα stimulation (Fig. 1B). Furthermore, D6 cells showed an enhanced response to TNFα-induced apoptosis, which restricted the defect to NF-κB activation and correlates with the anti-apoptotic function described for NF-κB (19, 20). Moreover, as IκBα is degraded and c-Rel is initially translocated upon TNFα stimulation with the same efficiency in Jurkat and clone D6, we postulated an intrinsic defect in c-Rel itself. Sequencing of the c-Rel gene present in clone D6 was evidence that clone D6 expressed a mutant version of c-Rel, characterized by a change of Ser residue at position 471 to an Asn residue (Fig. 5A). This position relies within the previously described transactivation domain of c-Rel (21, 22), suggesting that this mutant version of c-Rel may be unable to promote TNFα-dependent transcription. The experiments with Gal4DBD-c-Rel fusion proteins demonstrated that this mutation is indeed responsible for the TNFα-enhanced c-Rel transcriptional activity. Moreover, the identical response of wild type Gal4-c-Rel fusion construct transfectod either into mutant D6 or wild type Jurkat cells further confirmed that only c-Rel activation was impaired in clone D6. Furthermore, transfection of c-Rel wild-type into D6 cells partially recovered the wild-type phenotype, whereas overexpression of the c-Rel mutant S471N in Jurkat cells completely abolished TNFα-induced NFκB activity. Thus, this alteration in c-Rel was responsible for the lack of TNFα response observed in clone D6.

Although DNA binding of NF-κB complexes was detected in the nucleus in clone D6 as well as in Jurkat T cells upon stimulation by TNFα (Fig. 2A), there were striking differences related to the composition of the active NF-κB complexes bound to the NF-κB probe. In wild type cells, the NF-κB complexes were composed of either p65/p50 or c-Rel/p50 heterodimers as revealed by anti-p65 plus anti-c-Rel antibody pretreatment of the extracts. Both complexes were detected in the nucleus as soon as 30 min after TNFα stimulation, but at late times (4 h) the factors bound to the NF-κB probe were composed mainly of c-Rel in wild type Jurkat cells (Fig. 2C). The induction by TNFα of this c-Rel-containing complex at 4 h post-stimulation was absent in clone D6, indicating a failure of the later c-Rel induction upon TNFα stimulation. Once again, PMA plus ionophore stimulation triggered the activation of similar NF-κB binding complexes in the nucleus of both Jurkat and clone D6 cells (Fig. 2B), restricting again the defect to TNFα stimulation. These results fit the biphasic model of NF-κB binding activation by TNFα in T cells (1), in which there was an immediate nuclear translocation of p65 and c-Rel: p65 peaked immediately (30 min) but returned to basal levels after 4 h; in contrast, c-Rel was also induced by 30 min but was increasingly detected in the nucleus, reaching a plateau at 4 h that was maintained at least 15 h after stimulation. This progressive nuclear increase of c-Rel was absent in clone D6, whereas early p65 induction was similar than in Jurkat cells.

It has been described that different members of the IκB family can bind to NF-κB dimers and how these associations affect the NF-κB functionality (23). However, the failure to retain c-Rel in the nucleus observed in clone D6, once activated with TNFα, could not be attributed to a differential association of c-Rel with several IκB family members in Jurkat and clone D6 cells. In agreement with previous reports (23), we found both p65 and c-Rel predominantly bound to IκBα and IκBe; however, immunocomplexes with IκBo were also detected (Fig. 4A). Furthermore, IκBo was degraded with the same kinetics in Jurkat and clone D6, with no degradation of IκBβ and IκBe (Fig. 4B). Those results are intriguing considering that only a c-Rel point mutation seems to be responsible for TNFα unresponsiveness. However, they can be explained considering that c-Rel but not p65 gene transcription requires a TNFα-dependent NF-κB activation (1). Altogether these results suggest that pre-existing c-Rel was translocated to the nucleus, but since it is inactive, it was degraded or removed from the nucleus. Because NF-κB-dependent transcription is strongly depressed in the mutant, no new c-Rel is synthesized, so the overall result is that NF-κB-dependent response fades away. This supports the model where TNFα-induced NF-κB activity, in the context of T cell activation, mainly relies in c-Rel activation. This activation is controlled by an autocline loop: TNFα activation of c-Rel results in the transactivation of its own gene, further increasing NF-κB-dependent transcription. Furthermore, our...
results point to c-Rel as a key mediator of TNF-α-induced NF-κB-dependent activity. Thus, an inhibition of c-Rel abolishes this loop and results in a drastic overall effect of NF-κB-dependent transcription and, consequently, in function (i.e. increased apoptosis).

NF-κB activation by TNF-α involves the induced degradation of IκBa through the activation of the IκB kinase (24–27). However, a second level of regulation of the transcriptional activity has been described for other NF-κB family members, for example p65 (9, 12). This second level of regulation of p65 activity involves the phosphorylation of Ser-529 residue within the transactivation domain upon TNF-α stimulation, which enhances its transcriptional abilities. The drastic functional effect of c-Rel (S471N) mutation suggests that c-Rel is similarly subjected to a dual regulation by TNF-α. Thus, c-Rel activation at a first level involves IκB degradation and subsequent translocation to the nucleus, whereas a second level involves the phosphorylation of the transactivation domain, as described here. It is noteworthy that the Ser residue at position 471 is strictly necessary for TNF-α activation of c-Rel (Fig. 5A) but dispensable for PMA plus ionophore activation, as D6 cells respond to these stimuli as well as Jurkat cells. Similarly, the Gal4DBD-c-Rel 309–588 is equally activated by PMA plus ionophore regardless of whether it carries the mutation, indicating that either the PMA plus ionophore-induced activation of c-Rel relies on the phosphorylation of other residues (Fig. 5C) or that the phosphorylation defect is compensated with other signals. The fact that PMA plus ionophore induced a stronger phosphorylation of the wild type molecule will suggest the first possibility.

The kinase involved in this TNF-α-induced phosphorylation of Ser-471 is presently unclear. Several NF-κB family members, including IκBα, p50, p65, and c-Rel, have been shown to be phosphorylated, indicating a role of phosphorylation in functional activity. Thus, nuclear import of Drosophila protein Dorsal is regulated by phosphorylation (28). Moreover, recent results demonstrated that p65 transcriptional activity is augmented by a TNF-α-induced phosphorylation of Ser-529 (9). Phosphorylation of p65 also regulates its interaction with transcriptional machinery (8). On the other hand, the Ras and protein kinase Cζ-dependent phosphorylation of p65 in the N-terminal Rel homology domain was found to be essential for its transcriptional activity (11). Thus, protein kinase Cζ may have a dual role in NF-κB activation, as it has been also demonstrated to be a IκB kinase inducer (29). A Ras-dependent mechanism for activation of p65 that requires p38 MAPK activity independent of PI3K activation has been also described (10). In contrast, the p65 phosphorylation and subsequent activation induced by IL-1 has been recently shown to be dependent on PI3K activation (12). Moreover, the Ser/Thr kinase Akt (also known as protein kinase B), activated through lipid products of PI3K enzymatic activity, has been implicated in the regulation of NF-κB in Jurkat cells (30). However, it is unlikely that PI3K directly phosphorylates p65, since Akt stimulates IκB kinase activity and subsequent degradation of IκBα, so it is not the PI3K-stimulated activity that directly phosphorylates p65. On the other hand, c-Rel was demonstrated to be specifically phosphorylated in Ser residues (5). The MEKK1/JNK1 pathway has been shown to be implicated in NF-κB activity, and JNK1 has been found to be associated to c-Rel in vivo (31); however, JNK1 did not phosphorylated c-Rel in vitro, indicating a different kind of regulation and discarding JNK1 as a direct activating kinase.

We have shown that c-Rel is already phosphorylated in Jurkat cells, but TNFα further induces its phosphorylation (Fig. 7A). Solid phase assays using GST-c-Rel 421–588 as the substrate for phosphorylation by WCE demonstrated a strong basal phosphorylating activity in non-stimulated cells. However, phosphorylation of c-Rel was further increased after TNFα treatment. When a shorter recombinant protein spanning the residues 463 to 481 was used, only very low levels of basal phosphorylation could be detected, but a strong induced phosphorylation upon TNFα or PMA plus ionophore stimulation of the wild type protein was detected. This TNF-α-induced phosphorylation did not take place when the mutant was used, thus suggesting an essential role of Ser-471 phosphorylation for TNF-α-mediated activation of c-Rel (Fig. 7B). Although Ser-471 could be phosphorylated, our results do not exclude completely the possibility that it could be necessary for the binding of a specific kinase without being itself phosphorylated. PMA plus ionophore stimulation also induced a strong phosphorylation of the wild-type 464–481 fusion protein, suggesting that it was also able to activate the same or a different kinase capable of phosphorylating Ser-471. However, as mentioned above, the PMA plus ionophore-induced phosphorylation does not take place in mutant D6 c-Rel, albeit c-Rel was fully active in these cells. Although Ser-471 is essential for TNFα-induced c-Rel activity and seems to be phosphorylated, we have detected some TNF-α-induced phosphorylation in the transactivation domain of the S471N mutant protein. This result suggests that TNF-α activates one or several kinases that can phosphorylate c-Rel transactivation domain in vitro at different positions, not only in Ser-471. We have not succeeded in the identification of the kinase(s) involved in c-Rel phosphorylation by “in-gel” kinase assays (data not shown), which suggests that the kinase involved is not active in this kind of experiment.

On the other hand, it has been described that c-Rel and v-Rel interact with the basal transcriptional machinery factors TATA-binding protein (TBP) and transcription factor IIB through their transactivation domains (32). However, other reports show that TBP interaction relies exclusively on the first 50 amino acids of c-Rel, within the Rel homology domain (33). Mutation S471N abolishes a phosphorylation site in the transactivation domain that may be necessary for the interaction between co-factors required for TNF-α activation of c-Rel. It will be of interest to test whether Ser-471 is implicated in the interaction of c-Rel with the transcriptional machinery to understand the mechanism of TNF-α activation of c-Rel. Studies are in progress to elucidate this hypothesis.

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