A Point Mutation in NEMO Associated with Anhidrotic Ectodermal Dysplasia with Immunodeficiency Pathology Results in Destabilization of the Oligomer and Reduces Lipopolysaccharide- and Tumor Necrosis Factor-mediated NF-κB Activation*

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The NEMO (NF-κB essential modulator) protein plays a crucial role in the canonical NF-κB pathway as the regulatory component of the IKK (IκB kinase) complex. The human disease anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) has been recently linked to mutations in NEMO. We investigated the effect of an alanine to glycine substitution found in the NEMO polypeptide of an EDA-ID patient. This pathogenic mutation is located within the minimal oligomerization domain of the protein, which is required for the IKK activation in response to diverse stimuli. The mutation does not dramatically change the native-like state of the trimer, but temperature-induced unfolding studied by circular dichroism showed that it leads to an important loss in the oligomer stability. Furthermore, fluorescence studies showed that the tyrosine located in the adjacent zinc finger domain, which is possibly required for NEMO ubiquitination, exhibits an alteration in its spectral properties. This is probably due to a conformational change of this domain, providing evidence for a close interaction between the oligomerization domain and the zinc finger. In addition, functional complementation assays using NEMO-deficient pre-B and T lymphocytes showed that the pathogenic mutation reduced TNF-α and LPS-induced NF-κB activation by altering the assembly of the IKK complex. Altogether, our findings provide understanding as to how a single point mutation in NEMO leads to the observed EDA-ID phenotype in relation to the NEMO-dependent mechanism of IKK activation.

The NF-κB family of eucaryotic transcription factors plays a crucial role in immune and inflammatory responses, development, and control of apoptosis (1). In addition, several genetic diseases have been recently linked to defects in the NF-κB pathway activation, and some of them are associated with mutations in the gene encoding the NF-κB essential modulator (NEMO/IκKγ) protein (see Ref. 2 for a review). Amorphic mutations in NEMO cause incontinentia pigmenti, an X-linked dominant disorder with a variety of developmental abnormalities of the skin, teeth, hair, eyes, and central nervous system in heterozygous females and death in male fetuses (3). Most patients with incontinentia pigmenti carry a common DNA rearrangement resulting in truncation of the NEMO protein and complete suppression of NF-κB activation (4). Other pathologies are caused by NEMO mutations that impair but do not abolish NF-κB signaling from receptors required for ectodermal development and/or immune function. Among those, X-linked recessive anhidrotic ectodermal dysplasia with immunodeficiency (EDA-ID) has been associated with mutations in the coding region of the NEMO gene (5–7). The EDA developmental phenotype is characterized by rare conical teeth, sparse scalp hair, frontal bossing, and the absence or rarity of sweat glands. Male subjects with EDA-ID present EDA combined with life-threatening infections of multiple sites, including the digestive and respiratory tracts and the skin, the most common infectious agents being Gram-positive and -negative pyogenic bacteria as well as mycobacteria. A NEMO stop codon mutation causes a clinically more severe syndrome associating osteopetrosis and/or lymphoedema with EDA-ID (5). Recently, it has also been shown that hypomorphic NEMO mutations could cause immunodeficiency without ectodermal dysplasia (8, 9).

NEMO is part of the IKK (IκB kinase) complex, which also contains the protein kinases IKKa and IKKβ (10). Although NEMO does not possess a catalytic function, it is indispensable for signaling through the canonical NF-κB pathway, since cells not expressing the protein are totally unresponsive to all tested stimuli (11, 12). In the canonical pathway, proinflammatory stimuli lead to the activation of the IKK complex (13). The kinases then phosphorylate the inhibitory IκB proteins, which keep NF-κB transcription factors inactive in the cytoplasm of resting cells. IκBs are in turn polyubiquitinated and degraded by the proteasome, allowing NF-κB to enter the nucleus and activate gene transcription. The activation mechanism of the IKK complex is still unclear. NEMO functions as a signaling adaptor between the upstream regulators and the kinases, and its oligomerization has been shown to be required for the activation of the complex (14–17). Several studies have shown that phosphorylation (18, 19) and ubiquitination (20–23) of NEMO may also contribute to the regulation of IKK activation, but the precise role of these post-translational modifications remains to be elucidated.

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The NEMO molecule consists of several structural domains (Fig. 1A). The N-terminal half of the protein containing a large coiled-coil motif (CC1) is responsible for the interaction with IKK kinases (24, 25). The C-terminal domain (C-ter) is composed of a coiled-coil motif (CC2, aa 251–285), a leucine zipper (LZ; aa 301–337), and a zinc finger motif (ZF; aa 390–410) at the extreme C terminus. It is involved in signal perception (26) and in the oligomerization of the protein (27, 28). Several studies have shown that NEMO self-assembles into trimers (17, 24, 27), and we recently proposed that the minimal oligomerization domain of the protein consists of the CC2 and LZ coiled-coils interacting to form a six-α-helix bundle (29). Many pathogenic NEMO mutations are found in the C-ter domain (2). Whereas some are located in the CC2-LZ subdomain and may interfere with NEMO oligomerization, many of them are found within the zinc finger motif. The integrity of this motif has been shown to be important for full IKK activation in response to several stimuli, including TNF-α, LPS, IL-1 (26, 30), and UV radiation (31). The ZF motif may also be required for the ubiquitination of NEMO (21, 22, 32). However, it was also reported that EDA-ID mutations in the ZF impaired CD40 signaling without affecting the NF-κB activation by TNF and LPS (7), indicating that the role of this motif remains to be precisely determined.

In this paper, we studied the effects of an alanine to glycine substitution within the CC2 motif, which was identified in a patient with EDA-ID (5). We compared the biochemical properties of the purified C-ter fragments of NEMO, either wild-type (WT) or carrying the pathogenic mutation (A281G in murine NEMO). We show that this mutation destabilizes the association of the protein in trimer and induces a change in the conformation of the C-terminal zinc finger, indicating a communication between the zinc finger motif and the oligomerization domain. These subtle changes in biochemical properties correlate with a partial inhibition of the NF-κB pathway in response to several proinflammatory stimuli and with an alteration of the IKK complex assembly. These results allow us to discuss the mechanism of activation of the IKK complex by NEMO.

**EXPERIMENTAL PROCEDURES**

Reagents—n-Dodecylmaltoside (DDM) and n-octyl glucoside (OG) were from Roche Applied Science. LPS from Escherichia coli was purchased from Fluka (Serotype O111:B4), and human TNF-α was from Roche Applied Science. The absence of endotoxin contamination was checked in the RPMI 1640 medium (Invitrogen) and fetal calf serum (Biowest) used in cell culture.

**Construction, Expression, and Purification of Truncated Forms of NEMO**—Truncated forms of murine NEMO corresponding to its C-terminal half (hereafter called C-ter; aa 241–412), or to two shortened C-terminal domains named ΔZF (aa 241–387) and CC2-LZ (aa 251–337) lacking the zinc finger motif, were cloned into pET-24a and pET-28b (Novagen), respectively, as described previously (27, 29). The A281G mutation was introduced into the pET-24a/WT C-ter and the pET-28b/WT ΔZF constructions by using the QuikChange site-directed mutagenesis method (Stratagene). The sequences of the primers used for PCR amplification are available upon request. All nucleotide sequences were verified by DNA sequencing.

For the purification of the C-ter fragment bearing the wild-type sequence (WT C-ter) we started from 17 g of E. coli BL21(DE3)pLysS cells transformed with pET-24a/WT C-ter and grown at 30 °C in LB medium supplemented with 50 μg/ml kanamycin and 30 μg/ml chloramphenicol. At A600 = 1.5, 1 mM isopropyl-1-thio-β-D-galactopyranoside (Euromedex) was added to the medium for 3 h. All subsequent steps were conducted at 4 °C. Cells were harvested by centrifugation and washed in 100 mM Tris-Cl, pH 8.0, buffer containing 10 mM MgCl2 and 1 mM DTE. Cells were resuspended in Buffer A (50 mM MES, pH 6.0, 20 mM NaCl, 1 mM DTE, and 0.2 mM DDM) containing 5% glycerol and a protease inhibitor mixture (Complete, Roche Applied Science) and broken in a French press at 1,500 p.s.i. The lysate was then sonicated, diluted 2.5-fold in Buffer A, and centrifuged for 30 min at 12,000 × g. The supernatant was finally treated with 200 μg of RNase (DNase-free; Roche Applied Science) and 6,000 units of DNase I (RNase-free; Roche Applied Science) for 30 min at 37 °C before loading on a 42-ml Poros HS 20 column (2 × 12.8 cm; Applied Biosystems) preequilibrated in Buffer A. The bound material was eluted with a 400-ml linear gradient (20 mM to 1 mM NaCl). Fractions containing WT C-ter were pooled according to optical density profile and SDS-PAGE analysis and dialyzed against 40 mM Tris-Cl, pH 7.5, containing 20 mM NaCl, 0.2 mM DDM, and 1 mM DTE (Buffer B). Before applying the protein sample on a Resource S column (6 ml; GE Healthcare) equilibrated in Buffer A, the pH was carefully adjusted to 6.0 with 1 M MES, pH 6.0. Bound proteins were eluted with a 180-ml linear NaCl gradient (20 mM to 1 M NaCl) and fractions containing the WT C-ter protein were pooled and dialyzed against Buffer C (50 mM Tris-Cl, pH 8.0, 150 mM KCl, 0.2 mM DDM, and 1 mM DTE). The solution was further fractionated on a Resource Q column (6 ml; GE Healthcare) equilibrated in Buffer C. The flow-through fractions containing the pure WT C-ter protein were pooled, concentrated by ultrafiltration (Amicon Ultra-15; Millipore Corp.), and extensively dialyzed against Buffer C before freezing at −80 °C at a concentration of 1 mg/ml. The purification of the recombinant A281G C-ter protein was performed by using the same protocol, starting from a 4-liter culture of BL21-Gold (DE3) cells (Stratagene). The protein was frozen at a final concentration of 1.6 mg/ml. C-ter proteins were analyzed by MALDI-TOF MS on an ABI Voyager-DE™ STR mass spectrometer (AME Bioscience) after extensive dialysis in 10 mM HEPES, pH 7.5, 10 mM KCl, 0.1 mM DTE, and 0.1 mM DDM.

The His-tagged WT ΔZF and CC2-LZ proteins were purified as previously described (27). A281G ΔZF mutant protein was purified using the same protocol, yielding 18.2 mg of protein starting from 5.4 g of cells. All protein concentrations were determined either by the method of Bradford or by measuring A280 using extinction coefficients of 4,734, 3,555, and 2,368 M−1·cm−1 for the C-ter, ΔZF, and CC2-LZ proteins, respectively, calculated on the basis of their amino acid composition (33).

**Sedimentation Equilibrium Analysis**—Sedimentation equilibrium experiments were carried out at 4 °C in a Beckman Optima XL-A analytical ultracentrifuge (Beckman Instruments, Palo Alto, CA) equipped with 12-mm path length double sector cells in an An60 Ti rotor. Sedimentation was performed at 9,000, 14,000, and 18,000 rpm with WT and A281G C-ter at initial concentrations of 15.7 and 12.3 μM, respectively, in 20 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl, 10 mM OG, and 1 mM DTE. Cells were filled with 150-μl samples, giving a column height of 0.3–0.4 cm. Optical density was measured at 240 nm with a radial increment of 0.001 cm. Samples were allowed to equilibrate for 30 h, and equilibrium was verified by superimposing scans recorded at 90-min intervals. When equilibrium was reached, data were collected, and centrifugation speed was finally increased to 55,000 rpm for 10 h to sediment the proteins. Radial scans were then collected in each cell to obtain a base-line correction. The partial specific volumes (9) of WT and mutant C-ter at 4 °C, calculated from their amino acid composition, were 0.724 ± 0.005 and 0.723 ± 0.005 ml/g.
respectively (34). The calculated density of the buffer at 4 °C was 1.007 g/ml (34). Data were fitted with one-species, two-species, or multispecies models as described previously (27, 35).

Gel Filtration Analysis—The apparent Stokes’ radius of the C-ter proteins was determined at 4 °C using an AKTA Purifier 100 system equipped with a Superdex 75 HR 10/30 column (GE Healthcare). 500-µl samples at various protein concentrations (0.5 nM to 10 µM) were injected onto the column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 200 mM NaCl, 0.2 mM DDM, and 1 mM DTE (Buffer D) and developed at a constant flow rate of 0.5 ml/min. For each sample, the protein was diluted in Buffer D at the desired concentration and allowed to equilibrate at 4 °C. Depending on the loading concentration, sample elution was followed by measuring either the absorbance at 260 and 280 nm or the tyrosine fluorescence at 310 nm after excitation at 280 nm using a spectrophotofluorimeter RF-10AXL (Shimadzu) connected on-line to the AKTA system. Both detection methods yielded superimposable elution profiles. The elution of standard globular proteins and C-ter proteins is described in terms of Kav, which corresponds to the ratio (Ve – Vd)/(Vf – Vd), where Ve is the elution volume of the protein and Vf and Vd are the void and total volumes of the column determined by injection of blue dextran 2000 and 1,4-dithiothreitol, respectively. Bovine serum albumin (67 kDa, Kav = 35.2 Å), ovalbumin (43 kDa, Kav = 27.5 Å), chymotrypsinogen A (25 kDa, Kav = 20.9 Å), ribonuclease A (13.7 kDa, Kav = 16.4 Å), cytochrome c (12.4 kDa, Kav = 17.1 Å), and aprotinin (6.5 kDa, Kav = 12.5 Å) were used for calibration.

Circular Dichroism Spectroscopy—Far-UV CD measurements were performed on an Aviv 215 spectropolarimeter (Aviv Instruments, Lakewood, NJ) with samples extensively dialyzed against 10 mM sodium peroxide against 1 mM NaCl, pH 8.0, containing 200 mM NaCl, 0.2 mM DDM, and 1 mM DTE (Buffer D) and developed at a constant flow rate of 0.5 ml/min. For each sample, the protein was diluted in Buffer D at the desired concentration and allowed to equilibrate at 4 °C. Depending on the loading concentration, sample elution was followed by measuring either the absorbance at 260 and 280 nm or the tyrosine fluorescence at 310 nm after excitation at 280 nm using a spectrophotofluorimeter RF-10AXL (Shimadzu) connected on-line to the AKTA system. Both detection methods yielded superimposable elution profiles. The elution of standard globular proteins and C-ter proteins is described in terms of Kav, which corresponds to the ratio (Ve – Vd)/(Vf – Vd), where Ve is the elution volume of the protein and Vf and Vd are the void and total volumes of the column determined by injection of blue dextran 2000 and 1,4-dithiothreitol, respectively. Bovine serum albumin (67 kDa, Kav = 35.2 Å), ovalbumin (43 kDa, Kav = 27.5 Å), chymotrypsinogen A (25 kDa, Kav = 20.9 Å), ribonuclease A (13.7 kDa, Kav = 16.4 Å), cytochrome c (12.4 kDa, Kav = 17.1 Å), and aprotinin (6.5 kDa, Kav = 12.5 Å) were used for calibration.

Flame Atomic Absorption Spectroscopy—Zinc titration was performed as previously described (39) with samples of the C-ter proteins extensively dialyzed against a buffer containing 25 mM Tris-HCl, pH 8.0, 150 mM KCl, 0.2 mM DDM and 1 mM DTE. The 2A88G mutation corresponding to A281G in murine NEMO was introduced by using the QuickChange site-directed mutagenesis method (Stratagene) with primer sequences available on request. The 2A88G NEMO construct was inserted into a pcDNA3 vector as described previously (40). The A288G mutation corresponding to A281G in murine NEMO was introduced by using the QuickChange site-directed mutagenesis method (Stratagene) with primer sequences available on request. The 2.13E2 and 7oz/3 murine pre-B cells lines were maintained and transiently transfected as in a previous study (11). NEMO−/− Jurkat T cells (IM4.5.2 (41)) were grown in RPMI 1640 supplemented with 10% fetal calf serum and antibiotics. These cells were transiently transfected by a modified DEAE-dextran method similar to the one used for 1.3E2 cells. Briefly, cells were transfected with rhNEMO plasmid and 80 ng of WT or 2A88G NEMO construct were added per 106 cells. After 45 min at room temperature, cells were incubated in 10 ml of Tris-buffered saline buffer, washed and reincubated at 37 °C for 4 h. TNFα (10 ng/ml) for 4 h and finally lysed in a lysis buffer (25 mM Tris-phosphate, pH 7.8, 8 mM MgCl2, 1 mM dithiothreitol, 1% Triton X-100, 15% glycerol). Luminescence determination was carried out in a Berthold luminometer.

HeLa cells plated in 6-well dishes were transfected using jetPEI (Qiobio, Illkirch, France) according to the manufacturer’s instructions. After 24 h, cytoplasmic extracts were prepared as described previously (11) and analyzed with anti-NEMO polyclonal antibody 3328 (a kind gift of Dr. Nancy Rice).
containing 300 mM NaCl, 20 mM imidazole, 0.1 mM DTE, 1 mM DDM, and 5% glycerol were incubated for 1 h at room temperature with 100 μl of Ni²⁺-NTA magnetic agarose beads (Qiagen) equilibrated in the same buffer. The beads were then collected, washed twice in 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, 20 mM imidazole, 0.1 mM DTE, 1 mM DDM and 5% glycerol, and incubated for 1 h at room temperature with S100 extracts from wild-type 70Z/3 or NEMO²¹ pre-B cells (300 μg of proteins) in the same buffer supplemented with an EDTA-free protease inhibitor mixture (Complete; Roche Applied Science). Beads were washed twice in the latter buffer. Bound proteins were finally eluted with 40 μl of 20 mM sodium phosphate buffer, pH 7.0, containing 150 mM NaCl, 300 mM imidazole, 0.1 mM DTE, 1 mM DDM, and 5% glycerol and analyzed by Western blotting using an anti-IKK antibody (Imgenex).

RESULTS

Purification of Recombinant WT and Mutant Truncated Forms of NEMO—We performed biochemical studies using various fragments of the C-terminal half of the murine NEMO protein (Fig. 1A). The fragment called C-ter (from aa 241 to the C terminus), which is responsible for the regulatory role of the protein through connecting the IKK complex to different upstream activators (26), contains the minimal oligomerization domain of NEMO (27). Unlike the ΔZF and CC2-LZ proteins, the N terminus of the C-ter construct was not His-tagged (Fig. 1A) because of the possibility that the histidine residues would chelate the Zn²⁺ ion bound to the C-terminal finger. The missense C → G mutation at nucleotide 863 identified in a patient with EDA-ID leads to a Ala → Gly replacement at position 288 within the CC2 motif of human NEMO (5), which corresponds to residue 281 in murine NEMO (Fig. 1A). The same mutation was introduced in the nucleotide sequence of the C-ter protein and ΔZF constructs (see “Experimental Procedures”).

Whereas NEMO His-tagged subdomains were highly expressed as soluble proteins in E. coli at 37 °C, the C-ter fragment, either WT or carrying the A281G mutation, had to be expressed at lower temperature to limit the formation of inclusion bodies. All buffers were supplemented with the nonionic detergent DDM to prevent the formation of aggregates, as described for the purification of the recombinant full-length NEMO (27). The expression level of the C-ter fragments was very low, probably due to the zinc finger domain, since good expression levels were obtained with both ΔZF and CC2-LZ fragments. Since C-ter proteins were not expressed with an N-terminal His tag, our purification strategy consisted of three ion exchange chromatography steps (see “Experimental Procedures”). It yielded 1.3 and 1.7 mg of WT and A281G C-ter with a high degree of purity (Fig. 1B), starting from 17 and 11 g of cells, respectively. The integrity of both proteins was analyzed by MALDI-TOF MS, allowing us to obtain a Mr of 20,286.7 ± 1 Da and 20,272.5 ± 1 Da for WT and mutant C-ter, respectively. These values are identical to the calculated Mr assuming the proteolytic cleavage of the initial methionine residue. Despite the use of protease inhibitors, the WT and A281G ΔZF proteins were obtained along with a minor proteolytic fragment (Fig. 1B). Proteolysis occurred at the C terminus of the polypeptide within the poorly structured proline-rich region as shown.
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### TABLE 1

Sedimentation equilibrium and gel filtration data of the WT and mutant C-ter proteins

| Protein       | Initial protein concentration (µM) | Speed (rpm) | Single species mass (Da) | χ² | Monomer-trimer equilibrium | ΔG° (kcal/mol) | Kav (× 10^−10 M⁻¹) | Deduced molecular mass (Da) |
|---------------|-----------------------------------|-------------|--------------------------|----|----------------------------|----------------|----------------------|-----------------------------|
| WT C-ter      | 15.7                              | 14,000      | 29,550 ± 300             | 142| 4.4 ± 2.3                  | -11 ± 0.2      | 7.5 ± 0.1            | 63,847                      |
|               |                                   | 18,000      | 30,550 ± 200             | 333| 2.4 ± 0.4                  | 91             | 10.6 ± 0.1           |                             |
|               |                                   |             | 32,300 ± 300             | 211| 29 ± 23                    | 58             | 12 ± 0.3             | 8.5 ± 0.1                   |
|               |                                   |             | 30,250 ± 260             | 264| 4.9 ± 0.9                  | 34             | -11 ± 0.1            | 60,235                      |
| A281G C-ter   | 12.3                              | 14,000      | 29,550 ± 300             | 142| 4.4 ± 2.3                  | -11 ± 0.2      | 7.5 ± 0.1            | 63,847                      |
|               |                                   | 18,000      | 30,550 ± 200             | 333| 2.4 ± 0.4                  | 91             | 10.6 ± 0.1           |                             |
|               |                                   |             | 32,300 ± 300             | 211| 29 ± 23                    | 58             | 12 ± 0.3             | 8.5 ± 0.1                   |
|               |                                   |             | 30,250 ± 260             | 264| 4.9 ± 0.9                  | 34             | -11 ± 0.1            | 60,235                      |

by MALDI-TOF MS analysis and by Western blot using an antibody recognizing this region of the protein (data not shown). The apparent M_r values of the five recombinant fragments (Fig. 1B) were in agreement with the calculated ones.

### Sedimentation Equilibrium Analysis of WT and Mutant C-ter Domains

The oligomeric state of both WT and mutant C-ter domains was analyzed by sedimentation equilibrium at 4 °C in the buffer conditions used previously (27). Table 1 shows the data obtained at 14,000 and 18,000 rpm. A single species model did not correctly fit to C-ter protein radial distributions at 9,000 (not shown), 14,000, and 18,000 rpm. It yielded average molecular masses that did not correspond to either a monomeric, dimeric, or trimeric form, indicating an equilibrium between the monomer and multimeric species. The best fit of the data was obtained for both proteins with a model corresponding to a monomer/trimer equilibrium. The association equilibrium constant (K_a) and the corresponding free energy of binding (ΔG°) at 4 °C are given. The errors were deduced from the fitting curves. The use of a three-component model (monomer-dimer-trimer) did not improve the fit, so that the dimer will be a minor species (<4%). Binding experiments with 8-anilino-1-naphtalenesulfonic acid, a fluorescent probe for the molten globule state (42), did not show any difference between the two C-ter fragments (data not shown), suggesting that the A281G C-ter retained the same global fold as the WT protein.

### Mutant C-ter Shows Different Hydrodynamic Properties Compared with Its WT Counterpart

We next analyzed the two proteins by size exclusion chromatography at different concentrations in similar buffer and temperature conditions as above. Each sample was injected onto the column ~15 h after dilution of the protein at varying concentrations. At 10 µM WT and A281G, C-ter co-eluted in a single narrow peak with a partition coefficient (Kav) compatible with a globular trimeric species as deduced from the calibration of the column (Table 1 and arrow in Fig. 2A), in agreement with the sedimentation equilibrium data. However, when injecting samples at lower concentrations, chromatograms revealed major differences in the hydrodynamic properties of the two proteins. As shown in Fig. 2A, when injected onto the column at 1 µM, WT C-ter yielded a very broad peak reflecting the presence of different oligomeric species (solid line). In contrast, the mutant C-ter showed a less broad and almost symmetrical peak with an intermediate elution volume between that of the theoretical globular monomeric and trimeric species (dashed line). At decreasing protein concentrations from 1 to 0.1 µM, the elution profile of each protein retained the same form but shifted significantly toward higher elution volumes, corresponding to higher Kav values (Fig. 2A, inset), indicating a dissociation process. At these low concentrations, the absorbance was too weak, and the elution was followed by measuring the intrinsic fluorescence of tyrosine residues. At concentrations lower than 0.1 µM, the average Kav strongly dropped, as shown in Fig. 2A (inset), and at 1 nM and lower, the two proteins co-eluted in a single sharp peak (Fig. 2B), at a Kav of ~0.1. This peak was consistent with the presence of a single species, probably a monomer in an “extended” conformation (see “Discussion”).

In conclusion, although no difference in the quaternary structure of the WT and mutant C-terminal fragments could be seen in sedimentation equilibrium, gel filtration experiments reveal that the association/dissociation kinetics of the mutant C-ter differ from that of its WT counterpart, possibly due to a higher flexibility of the protein.

### Analysis of WT and Mutant C-ter Proteins by Circular Dichroism

We analyzed the consequences of the alanine to glycine mutation on the C-ter fragment secondary structures by far-UV CD spectroscopy. Fig. 3A shows the spectra obtained at 20 °C for the WT and A281G C-ter proteins at concentrations at which they exist as trimers. The two minima at 208 and 222 nm and the maximum at 191 nm are characteristic of coiled-coil proteins containing a high α-helix content (43). Deconvolution of the spectra to quantify the secondary structure content of the proteins yielded α-helical contents of 61.5 ± 0.3 and 56.7 ± 0.5% for the WT and mutant C-ter, respectively, revealing a significant loss of almost 5% of α-helices in the latter protein (Fig. 3A, inset). Both proteins had a low β-strand content (~7%), probably mostly due to the contribution of the C-terminal zinc finger (44). Moreover, the 222/208 ellipticity ratio, which can be used to estimate the coiled-coil character of an α-helical protein (45), was weaker for the mutant protein (Fig. 3A, inset), indicating a local disordered region within interhelical interfaces.

We next performed thermal denaturations of the C-ter proteins to provide information concerning the nature and cooperativity of the interactions stabilizing the trimers. For this purpose, we recorded the CD signal at 222 nm with 1 °C steps when increasing the temperature from 1 to 95 °C, followed by a final decrease to 25 °C. Little differences were observed when superimposing the spectra obtained at 25 °C in the heating and cooling steps (not shown), indicating that the unfolding process of the C-ter fragment can be considered as reversible. Fig. 3B shows the melting curves obtained for the WT and mutant C-ter at concentrations at which both proteins exist as trimers. Thermal unfold-
FIGURE 2. Gel filtration analysis of WT and mutant C-ter proteins at concentrations ranging from 10 μM to 1 nM. A, elution profiles at the loading concentration of 1 μM. WT C-ter yielded a different elution profile when injected at once (dotted line) than after 2 or 15 h of equilibration (solid line). In contrast, A281G C-ter yielded the same chromatogram at the three equilibration times (dashed line). The elution volumes ($V_e$) of the globular marker proteins, as well as of the theoretical globular monomeric ($\alpha_1$), dimeric ($\alpha_2$), and trimeric ($\alpha_3$) species of the C-ter proteins deduced from column calibration are shown. Arrow, elution volume of WT and mutant C-ter at the concentration of 10 μM (see Table 1). Inset, the average partition coefficient ($K_{av}$) is plotted against C-ter protein concentration. B, bovine serum albumin; Ov, ovalbumin; Ch, chymotrypsinogen A; R, ribonuclease A; C, cytochrome c. B, gel filtration of WT (solid line) and A281G (dashed line) C-ter at the loading concentration of 1 nM. Elution of the proteins was followed by measuring tyrosine fluorescence at 310 nm after excitation at 280 nm. a.u., arbitrary units.
FIGURE 3. Analysis of the secondary structure content and thermal stability of the WT and mutant C-ter proteins. A, far-UV CD spectra of WT (solid line) and A281G (dashed line) C-ter proteins. The spectra were recorded at 20 °C at the concentration of 12.2 and 15.0 μM for the WT and mutant C-ter, respectively. Inset, secondary structure contents deduced from the deconvolution of the spectra and 222/208 nm ratio reflecting coiled-coil interactions (45). α, α-helix; β, β-strand; r.c., random coil. B, thermal denaturation curves of the WT (black circles) and A281G (open circles) C-ter proteins at a concentration of 24.0 and 32.8 μM, respectively. Ellipticity was recorded at 222 nm and converted to differential extinction coefficient per residue (Δε). Data were fitted (solid lines) with a two-state model (see "Experimental Procedures"). C, thermal denaturation data of WT C-ter at the concentration of 2.1 (crosses), 12.2 (open diamonds), and 24.0 μM (open circles) fitted with a two-state model (solid lines). Inset, melting temperatures (Tm) and cooperativities (coop.) deduced from the fitting curves. D, thermal denaturation curves of A281G C-ter at a concentration of 3.2 (crosses), 15.0 (open diamonds), and 32.8 μM (open circles). Data were best fitted with a three-state model (solid lines). Inset, melting temperatures and cooperativities deduced from the fitting curves.
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ing transitions were highly cooperative, consistent with proteins forming ordered oligomeric structures. Both curves were correctly fitted with a two-state model, yielding different melting temperatures \( T_m \) of 41.7 and 36.8 °C (cooperativities of 9.1 and 6.2) for the WT and A281G C-ter, respectively. The mutant C-ter therefore appears significantly less stable than the WT protein. To address whether this single transition corresponded to the trimer dissociation, thermal denaturations were performed at different concentrations. A cooperative two-state model correctly fitted to the data obtained for the WT C-ter at all three protein concentrations (Fig. 3C). The melting temperature increased as the concentration increased from 2.1 to 12.2 μM (Fig. 3C, inset), confirming that the unfolding transition is tightly coupled to the trimer dissociation. A further concentration increase from 12.2 to 24 μM did not result in an increase of the \( T_m \) but only of the cooperativity of the transition. The concentration dependence for the thermal unfolding of the mutant C-ter protein was more complex (Fig. 3D). Indeed, CD thermal melts of this protein at the two lower concentrations could not be correctly fitted with a two-state model, suggesting the presence of a partially folded intermediate. Unfolding of the mutant could be modeled by a minimum of three states, which was confirmed by the fact that the first derivative of the transition curves at these concentrations revealed the presence of at least two transitions (data not shown). We also modeled the data at the higher concentration with a three-state model to be able to compare the \( T_m \) and cooperativity values (Fig. 3D, inset). The first transition showing a \( T_m \) between 20 and 30 °C appeared concentration-dependent and displayed a low cooperativity compared with the second transition. The latter took place at a \( T_m \) near 40 °C that seems to be independent of the protein concentration in the range from 3 to 33 μM.

To better characterize the unfolding of the proteins, several CD spectra were recorded during the thermal denaturation at intermediate protein concentrations (Fig. 4). When deconvoluting the mutant spectra from 1 to 35 °C corresponding to the first transition, we observed an important loss (~40%) in the α-helical content of the mutant C-ter as compared with only ~15% for the WT, the β-structure content remaining constant for both proteins. The spectra at 95 °C were superimposable and indicated some residual secondary structures in the C-ter proteins.

In conclusion, our detailed analysis of the CD properties of the WT and mutant C-ter proteins demonstrates a partial unfolding as well as a destabilization of the trimer of the pathogenic protein.

Fluorescence Analysis Reveals the Presence of a Tyrosinate in the Zinc Finger Motif—We finally analyzed recombinant proteins by fluorescence spectroscopy. The C-ter fragment contains four tyrosine residues distributed all along the molecule, as shown in Fig. 1A, and four phenylalanines but no tryptophan. Its intrinsic fluorescence will therefore be entirely due to tyrosines, since the phenylalanine contribution is negligible. Emission spectra of the WT and A281G C-ter recorded after excitation at 278 nm were very different (Fig. 5A). The mutant protein yielded a spectrum mostly characteristic of tyrosine residues with a maximum at 306 nm, whereas the WT C-ter spectrum consisted in a broad peak with a maximum shifted to 316 nm and a strong shoulder near 345 nm. Note that a shoulder at 345 nm is also visible in the spectrum of the mutant C-ter. The fluorescence quantum yield of the A281G C-ter (0.063) appeared slightly higher than that of the WT protein (0.058). Surprisingly, both proteins displayed similar excitation spectra (data not shown). Indeed, when the emission wavelength was set to 306 or 345 nm, a maximum at 280 nm or at 284 nm with a marked shoulder at 290 nm was observed, respectively, revealing the presence of two populations of fluorophores. The abnormal signal around 345 nm could either come from contaminating proteins containing tryptophan residues or come from a tyrosinate known to display fluorescent properties similar to that of a tryptophan (46). The high degree of purity obtained for the C-ter proteins (Fig. 1B) suggested that the abnormal signal was not likely to arise from a tryptophan-containing contaminant, and their \( M_w \) determined by MALDI-TOF MS indicated that the tyrosines were not posttranslationally modified. In addition, the fluorescence emission properties of the recombinant WT and mutant ΔZF as well as CC2-LZ proteins yielded emission spectra characteristic of tyrosine-containing proteins with no shoulder at 345 nm (Fig. 5B). Since previous results did not indicate any important change in the conformation of the oligomerization domain upon deletion of the ZF (29), this suggested that the signal at 345 nm could be due to the tyrosine residue located in the zinc finger motif. The quantum yields of the three proteins were similar (~0.030) and corresponded to about 50% of the value for the entire C-ter proteins, indicating that the tyrosine situated in the ZF contributes significantly to the fluorescence of the entire C-terminal fragment. To address whether an emission spectrum typical of tyrosines could be recovered upon protein denaturation, we placed C-ter proteins in stringent denaturing conditions (6 M GdmCl, 10 mM EDTA to chelate the zinc ion and at pH 2.0) to make sure to unfold the ZF, which is known to be a highly stable domain. As illustrated in Fig. 5C (left), we observed a strong decrease in the intensity of the signal at 345 nm upon excitation at 278 nm. This signal even disappeared for the mutant protein when irradiated at 278 or 290 nm (not shown). Fluorescence intensity of N-acetyltryptophanamide decreased by ~30% when lowering the pH from 8.0 to 2.0 (data not shown) compared with a decrease of ~75% for the signal at 345 nm. Thus, this signal is unlikely to arise from traces of tryptophan containing impurities but is rather due to a tyrosine that emits a tyrosinate signal dependent on the intrinsic conformation of the zinc finger. This signal did not disappear when placing C-ter proteins in the presence of 6 M GdmCl only (Fig. 5C, right) and was even more marked for the WT protein, the spectrum of which then displayed two emission peaks with maxima at 305 and 345 nm. Under these conditions, CC2-LZ will unfold, whereas the zinc finger motif could retain its conformation due to the highly stable coordination bonds (47), explaining the difference in the spectra shown in the two panels of Fig. 5C. This suggests that the tyrosinate signal may arise from the fluorescence properties of the tyrosine within the ZF itself rather than from a simple fluorescence transfer from the other tyrosines in the CC2-LZ. The tyrosinate emission could result from direct excitation of ground state ionized tyrosine or from excited state proton transfer from singlet excited tryptophane, since the pKₐ of the phenol group decreases from 10.3 in the ground state to 4.2 in the excited state. We observed no change in the tyrosinate excitation spectrum for both WT and A281G C-ter in a pH range from 5.9 to 8.3 below the theoretical pKₐ of a ground state tyrosine (data not shown), suggesting that the emission band at 345 nm may rather originate from an excited state tyrosinate (48).

To date, NEMO zinc finger has been identified only on the basis of sequence homology. We analyzed WT and mutant C-ter proteins by flame atomic absorption spectroscopy, which allowed us to show for the first time that both proteins actually chelated zinc. The deduced zinc/protein stoichiometry was 0.48 ± 0.15 and 0.26 ± 0.15 for the WT and mutant C-ter, respectively (data not shown), which is significant although smaller than the expected value of 1 (see “Discussion”). Taken together, our results in fluorescence spectroscopy reveal the presence of a tyrosinate forming in the environment of the C-terminal zinc finger of WT NEMO. The weaker tyrosinate signal in the mutant C-ter protein indicates a small change in the conformation of the tyrosine residue located in the ZF, suggesting an internal communication between the
FIGURE 4. Far-UV CD spectra as a function of temperature. A, CD spectra of WT C-ter at a concentration of 12.2 μM. B, CD spectra of mutant C-ter at the concentration of 15.0 μM.

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minimal oligomerization domain where the mutation is located and the zinc finger.

**Mutant Human NEMO Does Not Fully Restore TNF-α- and LPS-induced NF-κB Activation upon Complementation of NEMO(−) Cell Lines**—We compared the activation of the NF-κB pathway mediated by the full-length WT and pathogenic NEMO proteins in cells. For this purpose, different NEMO-deficient cell lines were transiently co-transfected with an Igκ-luciferase reporter plasmid and the human cDNA (NEMO), either WT or carrying the A288G mutation. Pre-B 1.3E2 cells (11) were used to test the NF-κB activation in response to LPS but could not be used for the TNF-α, since they lack the required receptors. We therefore used NEMO−/− Jurkat T cells (JM4.5.2) to investigate TNF-mediated NF-κB activation. As shown in Fig. 6A, the pathogenic protein did not completely restore the NF-κB activation in response to both LPS

**FIGURE 5. Fluorescence analysis of recombinant proteins.** A, emission spectra of the WT (solid line) and mutant (dashed line) C-ter proteins at a concentration of 15 μM after excitation at 278 nm. a.u., arbitrary units. B, emission spectra (excitation at 278 nm) of the WT (solid line) and A281G (dashed line) ΔZF and of the CC2-LZ (dotted line) proteins at a concentration of 15 μM. C, emission spectra (excitation at 278 nm) of the WT and mutant C-ter at 2 μM in the presence of 6 M GdmCl, 10 mM EDTA at pH 2.0 (left) or 6 M GdmCl only (right).
and TNF-α, displaying only 75–80% of activity compared with the wild-type protein. This difference was significant and reproducible. We verified by transient transfection of HeLa cells with different amounts of the constructs that the levels of expression of the WT and mutant proteins were similar (Fig. 6B). These results show that the pathogenic mutation affects several proinflammatory stimuli leading to the activation of NF-κB transcription factors.

The Pathogenic Mutation Interferes with the Assembly of the IKK Complex through the Impairment of NEMO Oligomerization—Since the pathogenic mutation leads to the oligomeric instability of NEMO, we wondered whether the EDA-ID mutation could interfere with the assembly of the IKK complex. Cytoplasmic S100 extracts were prepared either from wild-type pre-B cell line (70Z/3), which contains the native endogenous NEMO associated to IKK kinases, or from the 1.3E2 NEMO-deficient cells as a negative control. Recombinant His-tagged WT and A281G ΔZF proteins captured on Ni²⁺-NTA beads were then incubated in S100 extracts from both cell types, and the interaction with IKK complex was detected by Western blotting with an anti-IKK antibody after elution of the His-tagged proteins. As shown in Fig. 7A, specific interactions of ΔZF proteins with the IKK complex were detected in 70Z/3 cells, whereas no association was observed in 1.3E2 NEMO-deficient cells, indicating that the interaction with the IKK complex results from NEMO oligomerization. In addition, when we compared the recovery of IKKα bound to the His-tagged ΔZF fragments, we observed that the A281G protein allowed us to fish less IKKα than the WT one. This was not due to a different amount of His-tagged

FIGURE 6. Incidence of the pathogenic mutation on NF-κB activation. A, transient transfections. 1.3E2 and JM4.5.2 NEMO-deficient cells were co-transfected with the reporter plasmid Igκ-luciferase and with an expression vector encoding either WT (WT) or A288G mutant (A/G) human NEMO. 1.3E2 and JM4.5.2 cells were stimulated for 4 h with 15 μg/ml LPS and 10 ng/ml TNF-α, respectively. The NF-κB pathway activation corresponding to the ratio of luciferase activity in cell extracts in the presence and absence of the stimulus is taken as 100% activity for WT human NEMO. Absolute activation values for WT NEMO were 33 ± 7-fold for LPS and 11 ± 1.5-fold for TNF-α. The error bars represent the S.D. values over three experiments. B, Western blot showing human NEMO (hNEMO) expression level in extracts of transiently transfected HeLa cells. Note that the endogenous NEMO protein was detected in mock-transfected cells. Each transfection was made in duplicate.
WT and A281G proteins bound to the beads, since SDS-PAGE analysis with Coomassie staining revealed that this amount was similar (Fig. 7B). Thus, the oligomeric instability of NEMO caused by the pathogenic mutation interferes with the assembly of the IKK complex, accounting for the reduced NF-κB activation.

DISCUSSION

Several genetic diseases have been recently linked to mutations in the gene encoding the NEMO protein required for signaling through the canonical NF-κB pathway (2). In particular, EDA-ID has been associated with hypomorphic NEMO mutations, leading to a reduced but not abolished NF-κB activation (5). Disease variability among EDA-ID patients is likely to partly result from the hypomorphic nature of these mutations. In this context, studying their effect on structure-function relationships of NEMO could possibly help to better understand both the pathology itself and the mechanism of IKK activation by NEMO. In this work, we used several biochemical and biophysical methods to investigate the effect of an Ala→Gly pathogenic mutation located in the CC2 coiled-coil on NEMO properties. Our study focused on the C-terminal domain of NEMO, called C-ter, which is involved in the oligomerization of the protein as well as in its regulation of the IKK complex activation (26, 27).

Both WT and mutant C-ter proteins form globular and compact trimers as judged by sedimentation equilibrium and gel filtration at the protein concentration range from 4 to 100 μM. The mutant exhibits no tendency to aggregate, a good stability, a low affinity for 8-anilino-1-naphtalenesulfonic acid, and a cooperative thermal denaturation transition, indicating that the pathogenic mutation leads to a native-like state of NEMO C-terminal domain. However, a detailed study by gel filtration at concentrations in the micromolar to nanomolar range showed strong differences in the hydrodynamic behavior of the WT and mutant C-ter. Both proteins yielded a broad peak at concentrations at which sedimentation equilibrium data show the presence of both trimeric and monomeric species (Fig. 2A), suggesting that the interconversion between these species is slow as compared with the column retention time. Furthermore, when aliquots were applied onto the column at different times following dilution initiating dissociation, the elution profiles differed for the WT, whereas they were superimposable for the mutant (Fig. 2A). This suggests a difference in the interconversion rate constants or the existence of a different kinetic intermediate. The latter possibility can be ruled out in the case of the WT protein, because its thermal denaturation follows a simple two-state transition (Fig. 3C). The observed differences in the elution profiles are therefore likely to be due to a change in the monomer/trimer interconversion rate constants. Since CD thermal denaturation showed that the mutant trimer is less stable than the WT (Fig. 3B), the dissociation rate constant (k_diss) may be more specifically affected.

The fact that at a very low concentration, where both proteins should be completely dissociated according to sedimentation equilibrium, the hydrodynamic radius of the proteins became larger and was surprisingly similar to that of a globular trimer (Fig. 2B) deserves comment. Because gel filtration measures the compactness of a protein species (49), this form may correspond to a monomer in an expanded state. This cannot be an aggregated monomer, since it would then be eluted in the void volume and since we observed an increase of this species upon dilution of the proteins. We previously showed, using synthetic peptides, that CC2 and LZ coiled-coil subdomains interact with each other, forming a six α-helix bundle (29). It is then tempting to speculate that the expanded monomer may not contain any contacts between the CC2 and LZ subdomains, whereas the compact monomer may correspond to an antiparallel coiled-coil of the CC2 and LZ motifs.

A clear difference between the WT and mutant proteins was also revealed by our extensive study of their CD properties. Indeed, comparative analysis of far-UV CD spectra revealed a significant loss of 4.8% in the α-helical content of the mutant C-ter trimer compared with the WT. This is possibly due to a local disordered region surrounding the glycine residue situated at a heptad position in the CC2 coiled-coil. In addition to the importance of core a and d positions for the structural stability of α-helical coiled-coils (50), it is well known that glycine has more conformational freedom and is inherently more destabilizing than alanine when its position is not at the end of an α-helix (51). This loss in the α-helical content indicates that the Ala→Gly substitution induces a helix-coil transition for 8 residues, corresponding to a loss of about two turns of the α-helix. This disordered cluster effect is important, since the unfolding/dissociation transition is about 7 °C lower for the mutant than the WT. This cooperative destabilizing effect of glycine could initiate a stronger fluctuation of other side chains within the coiled-coil interface, leading to the cooperative separation of the three subunits of the entire C-ter domain. These data are fully consistent with our previously proposed structural model for the minimal oligomerization domain consisting of a six-helix bundle with an inner, trimeric, coiled-coil core of CC2 motifs (29).

CD thermal denaturation at low protein concentrations clearly shows the presence of a second transition for the mutant at a lower melting temperature, around 25 °C. This transition, corresponding to a drop of about 40% of helicity at 15 μM, is concentration-dependent. Since it cannot be assigned to the transition generally attributed in coiled-coils to end-fraying, which is an unimolecular process (52), the question then arises about the origin of this second transition. One possibility could be that it reflects the presence of a dimeric unstable intermediate as previously observed upon thermal unfolding of a trimeric coiled-coil (53). However, a model including a monomer-dimer-trimer equilibrium did not improve the fit of sedimentation equilibrium data, so that in the concentration range from 4 to 100 μM, the dimer is a minor species (<4%). Given the packing defect around the Gly residues in the mutant trimer, this transition may thus be attributed to the dissociation of a
second population of trimer. As the temperature increases, this trimeric species may start fraying at the C terminus around the glycine due to weaker α-α′ interactions. This may destabilize the C terminus of the CC2 coiled-coil sufficiently for the dissociation to occur at a lower temperature. This model that considers the existence of two trimer species is consistent with the sedimentation equilibrium results. It should also be noted that CD spectra of both proteins at 4 °C were almost superimposable, which could explain why we did not see any difference in the trimeric association constant of C-ter proteins in sedimentation equilibrium experiments that were done at this temperature.

Fluorescence studies revealed an abnormal fluorescence emission at 345 nm upon excitation at 278 nm for the WT C-ter. This signal is unlikely to arise from a tryptophan-containing contaminant, considering the degree of purity of the protein (Fig. 1B) and because it almost disappears in stringent denaturing conditions (Fig. 5C). Our data rather suggest that the emission at 345 nm results from the tyrosine residue located in the C-terminal zinc finger that fluoresces as a tyrosinate (46). A model of NEMO zinc finger domain showing the surrounding environment of this tyrosine is shown in Fig. 8. This model is based on the NMR structure of the ninth zinc finger found within the U-shaped protein, which was shown to mediate protein/protein rather than protein/DNA interactions (47, 54). NEMO ZF contains the three most conserved hydrophobic residues, Phe405, Tyr402, and Leu410 (human numbering) that are known to be critical to the packing of the finger.

FIGURE 8. NEMO zinc finger modeling. A, schematic representation of NEMO C-terminal zinc finger. “CCHC” residues involved in metal coordination are shown in gray circles. Important conserved hydrophobic residues are shown in boldface circles. Amino acid numbering refers to the human protein. B, molecular modeling of NEMO ZF based on the NMR structure of the ninth zinc finger from the Drosophila FOG family protein U-shaped (Protein Data Bank entry 1FU9) (54). The Insight II program (Accelrys, San Diego, CA) was used. The zinc ion is represented as a gray ball, chelating CCHC residues are light gray, and the tyrosine residue is dark gray.
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(55) (Fig. 8A). The consensus aromatic side chain (Tyr<sup>402</sup>) is at the juxtaposition of the three architectural elements constituted by the N-terminal β-hairpin, the C-terminal α-helix, and the tetrahedral Zn<sup>2+</sup>−binding site (Fig. 8B) and is likely to be involved in a staggered horizontal stacking with the histidine ligand His<sup>413</sup> (55). It has been shown that this aromatic side chain plays an important role in the thermodynamic stability of a zinc finger motif but not in the affinity for the metal ion (56). Moreover, the important role of this side chain is illustrated by the fact that its substitution by a leucine in the human tumor suppressor protein WT1 was linked to the Frasier syndrome (57). In NEMO, the orientation of Tyr<sup>402</sup> and its proximity to His<sup>413</sup> make possible an hydrogen bond between its hydroxyl group and the nitrogen atom of His<sup>413</sup> not implicated in the coordination of the zinc ion. This bond could contribute to the excited state protolysis.

In addition to the role of this aromatic side chain in the dynamic stability of a zinc finger, its fluorescence properties in NEMO can constitute a spectral probe to monitor its conformation in the ZF. The weak tyrosinate signal in the mutant C-ter suggests a slight change in the conformation of the tyrosine residue and possibly in the thermodynamic stability of the ZF motif. This change was not due to a difference in the zinc content, since both proteins show similar stoichiometries by flame atomic absorption spectroscopy, although lower than the theoretical value of 1. This is probably due to a partial loss of zinc upon extensive dialysis against an EDTA-containing buffer. Since the pathogenic mutation is located in the CC2 coiled-coil, our results bring to evidence a close intercommunication between the minimal oligomerization domain of NEMO and its zinc finger. The existence of a contact between these domains was recently verified by comparing the CD thermal denaturation transitions of the CC2-LZ domain and the entire C-ter. Indeed, the zinc finger has a stabilizing effect on the CC2-LZ, since it increases the melting temperature of the entire C-ter by about 8 °C.<sup>4</sup>

Finally, we determined to what extent the pathogenic mutation altered the activation of the NF-κB pathway in cells. Monogenic diseases that are caused by "loss of function" of a single protein often result from rapid degradation or accelerated turnover of the mutant protein by cellular proteases as a consequence of misfolded or damaged polypeptides or impaired cellular trafficking (58). In the case of the EDA-ID mutation, we can exclude this hypothesis, since the intracellular steady-state concentration of the mutant is similar to that of the WT NEMO protein in HeLa cells (Fig. 6B). Despite its correct expression, the human mutant protein (A288G) does not fully restore LPS- and TNF-induced NF-κB activation in NEMO-deficient murine pre-B and human T lymphocytes, respectively. The activation level of the NF-κB pathway was reduced by 20−25%, which has been shown to be sufficient to cause the EDA-ID phenotype in case of other mutations (2). The more severe pathology called incontinentia pigmenti, which is lethal for males, has also been associated with NEMO mutations (3). However, some of these mutations exhibit up to 40% of residual NF-κB activity using similar functional complementation assays.<sup>5</sup> This points out the complexity to establish a genotype/phenotype correlation of NEMO-related pathologies. In addition, we cannot rule out the possibility that NF-κB activation in response to LPS and TNF-α could be more affected by the A288G mutation in other cell types. Moreover, other signaling cascades triggering NF-κB activation, such as IL-1β, IL-18, and CD40L could be defective as well. It should also be noted that in this study the consistent link between the EDA-ID phenotype and the NF-κB alteration has been established with cell lines that do not derive from fibroblasts of the patient. The particular molecular chaperone environment could differ in the patient cells and then affect the NF-κB signaling pathway to a different extent.

The pivotal role of the IKK complex demands that the NEMO protein recognizes and presumably integrates diverse signaling cascades through its C-terminal regulatory domain. The structural and biochemical bases underlying IKK activation by NEMO remain poorly characterized. Although it is clear that phosphorylation in the activation loop of the kinases is required, the NEMO-dependent mechanism regulating these phosphorylation events remains to be established. NEMO oligomerization has been shown to be required for IKK activation (14−17). Using pure WT or mutant ΔZF as bait to fish the IkB kinases, we show that the destabilizing A288G mutation is likely to interfere with the assembly of the IKK complex through the impairment of oligomerization (Fig. 7). A recent and attractive mechanism relies on a small fraction of NEMO, which becomes Lys<sup>63</sup>-polyubiquitinated in IL-1B (59), TNF-α (21), genotoxic stress (60), NOD2/RIP2 (23), and T-cell receptor (22) signaling. Interestingly, a site of Lys<sup>63</sup>-polyubiquitination of NEMO was characterized by mass spectrometry in NOD2/RIP2 signaling following stimulation with the peptidoglycan breakdown product MDP and corresponds to Lys<sup>286</sup>, which is very close to the A288G mutation (23). This ubiquitination site is likely to be located in the locally disordered region of the CC2 coiled-coil revealed by our CD data of the pathogenic mutant, suggesting that the EDA-ID mutation could also interfere with NEMO ubiquitination. However, no direct validation for this hypothesis is available, since when we coexpressed the WT or A288G mutant protein with hemagglutinin-tagged ubiquitin in 293T or when we stably reconstituted NEMO-deficient JM4.5.2 cells with the WT or mutant protein following TNF-α stimulation, the polyubiquitination pattern of the mutant was similar to that of the WT (data not shown).

Whatever the NEMO-dependent model of the IKK activation by oligomerization or ubiquitination or by a concerted mechanism, several previous genetic studies reported the functional importance of the minimal oligomerization domain (15−17, 28, 29, 61) as well as of the ZF motif (26, 30, 31). Clearly, the structural integrity of these two domains should be preserved to allow a full NF-κB activation. In the present study, we showed in vitro by fluorescence and far-UV CD spectroscopies that the A288G pathogenic mutation alters both NEMO trimerization and the conformation of the C-terminal zinc finger. The dual effect of this mutation could contribute to the loss of NEMO function in a cooperative manner and could explain why the lack of a single methyl group induces such an alteration of the NF-κB pathway, causing the EDA-ID pathology. In light of these in vitro studies, we conclude that the reduced NF-κB activation by LPS and TNF-α caused by the EDA-ID A288G mutation is essentially due to a defect in NEMO oligomerization and possibly to a change in the ZF conformation that could interfere with the assembly of upstream yet unknown signaling molecules.

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<sup>4</sup>F. Agou, unpublished data.

<sup>5</sup>G. Courtois, unpublished results.
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