Myotrophin/V-1, a Protein Up-regulated in the Failing Human Heart and in Postnatal Cerebellum, Converts NFκB p50-p65 Heterodimers to p50-p50 and p65-p65 Homodimers*

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Myotrophin/V-1 is a cytosolic protein found at elevated levels in failing human hearts and in postnatal cerebellum. We have previously shown that it disrupts nuclear factor of κB (NFκB)-DNA complexes in vitro. In this study, we demonstrated that in HeLa cells native myotrophin/V-1 is predominantly present in the cytoplasm and translocates to the nucleus during sustained NFκB activation. Three-dimensional alignment studies indicate that myotrophin/V-1 resembles a truncated IκBα without the signal response domain (SRD) and PEST domains. Co-immunoprecipitation studies reveal that myotrophin/V-1 interacts with NFκB proteins in vitro; however, it remains physically associated only with p65 and c-Rel proteins in vivo during NFκB activation. In vitro studies indicate that myotrophin/V-1 can promote the formation of p50-p50 homodimers from monomeric p50 proteins and can convert the preformed p50-p65 heterodimers into p50-p50 and p65-p65 homodimers. Furthermore, adenovirus-mediated overexpression of myotrophin/V-1 resulted in elevated levels of both p50-p50 and p65-p65 homodimers exceeding the levels of p50-p65 heterodimers compared with Adβgal-infected cells, where the levels of p50-p65 heterodimers exceeded the levels of p50-p50 and p65-p65 homodimers. Thus, overexpression of myotrophin/V-1 during NFκB activation resulted in a qualitative shift by quantitatively reducing the level of transactivating heterodimers while elevating the levels of repressive p50-p50 homodimers. Correspondingly, overexpression of myotrophin/V-1 resulted in significantly reduced NFκB-luciferase reporter activity. Because myotrophin/V-1 is found at elevated levels during NFκB activation in postnatal cerebellum and in failing human hearts, this study cumulatively suggests that myotrophin/V-1 is a regulatory protein for modulating the levels of activated NFκB dimers during this period.

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Myotrophin/V-1 (Myo/V1)1 protein was initially characterized in the mammalian heart, where it was called myotrophin (1), and in the rat cerebellum, where it was called V-1 (2). It was later found to be ubiquitously expressed in all mammalian tissues (3, 4). Myo/V1 is a 12-kDa ankyrin repeat-containing intracellular protein that has been found at elevated levels in failing human hearts (5) as well as in the hearts of spontaneously hypertensive rats (6). Although Myo/V1 was originally described as a trophic protein (myotrophin) exhibiting growth properties exogenously on rat neonatal myocytes (1), other studies showed that this protein was only present in intracellular space (2, 7–9) and its trophic growth properties on neonatal myocytes were not confirmed (10). Moreover, this protein was originally identified and isolated only from an intracellular location (1), and a transcriptional regulatory function has been proposed (3, 9).

Since its discovery, investigators have proposed various functions for Myo/V1 protein (1–3). In the postnatal rat cerebellum, the cellular level of soluble Myo/V1 was found to be transiently up-regulated immediately after birth and later declined displaying a unique pattern of expression among 120 soluble proteins, implicating its role during postnatal cerebellum development (2). Because of its aberrant expression in genetically defective cerebellar granular cells, this protein was proposed to play a role in granular cell differentiation process (Unigene Mm.4123) (7). To date, however, the molecular function of Myo/V1 protein is still lacking. We recently reported that Myo/V1 protein exhibits significant homology to IκBα protein and that Myo/V1 can disrupt the NFκB-DNA complexes in vitro (3). Utilizing our recombinant Myo/V1 protein, the NMR structure of Myo/V1 was determined (11, 12), and the ankyrin repeats of Myo/V1 exhibited structural features similar to those of IκBα (13, 14) at the three-dimensional level.

In response to a variety of pathophysiological and developmental signals, the NFκB/Rel family of transcription factors are activated and form different types of hetero- and homodimers among themselves to regulate the expression of target genes containing κB-specific binding sites (15, 16). Among the activated NFκB dimers, the p50-p65 heterodimers are known to be involved in enhancing the transcription of target genes and the p50-p50 homodimers in transcriptional repression (17–22). However, the p65-p65 homodimers are known for

1 The abbreviations used are: Myo/V1, myotrophin or V-1; TNF, tumor necrosis factor-α; CHX, cycloheximide; GSA, gel-shift assay; NFκB, nuclear factor of κB; IκBα, inhibitor of κB-α; Tricine, N-tris(hydroxy-methyl)glycine; aa, amino acid(s); PTTc, fluorescein isothiocyanate; DAPI, 4,6-diamidino-2-phenylindole; SRD, signal response domain; oligo, oligonucleotide; NLS, nuclear localization signal; NIK, NFκB-inducing kinase; IKK, IκBα kinase.

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both transcriptional activation and repressive activity against target genes (23–31). NFkB activation is regulated at multiple levels. The dynamic shuttling of the inactive NFkB dimers between the cytoplasm and nucleus by IxB proteins (32–35) and its termination by phosphorylation and proteasomal degradation (36, 37), direct phosphorylation (38), acetylation of NFkB factors (39), and dynamic reorganization of NFkB subunits among the activated NFkB dimers (40–42) have all been identified as key regulatory steps in NFkB-mediated transcription process. αB DNA binding sites with varied affinities to different NFkB dimers (43) have been discovered in the promoters of several eukaryotic genes (16, 20, 22, 44, 45), and the balance between activated NFkB homeo- and heterodimers ultimately determines the nature and level of gene expression within the cell (18, 22). However, thus far the underlying molecular mechanism for the generation and dynamic reorganization of NFkB dimers during chronic activation is unknown.

Here, for the first time, we show that Myo/V1 acts as a “zipper chaperone” protein to generate NFkB homodimers from monomeric p50 proteins and with its “unzipping” function converts the transcriptionally active p50-p65 heterodimers to transcriptionally repressive dimers in HeLa cells, thus attenuating NFkB-mediated transcription.

**EXPERIMENTAL PROCEDURES**

**Recombinant Expression Plasmids and Adenoviruses—**Recombinant Myo/V1 protein was expressed in *Esherichia coli* in two different forms (~12-kDa full-length and histidine-tagged ~14-kDa fusion protein) using pET expression vectors as described before (3). The following mammalian expression plasmids were constructed by recombinant DNA methods. For pDNA-AM1.1-Myo/V1, because of the poor translation initiation Kozak site in the Myo/V1 mRNA, we engineered a heterologous highly efficient Kozak site into 5’-untranslated region of Myo/V1 so that Myo/V1 is expressed at high levels in mammalian cells. In *vitro* transcription and translation with pcDNA-AM1.1-Myo/V1 template DNA confirmed the synthesis of 12-kDa Myo/V1 protein at higher levels than the native Myo/V1 mRNA (data not shown). For pc-Btk-tk-luc, the parent chloramphenicol acetyltransferase reporter plasmid containing a minimal thymidine kinase promoter and two αB enhancer sites was obtained and replaced with the coding region of luciferase enzyme. pRSV-RelA vector expressing p65 was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. The expression plasmid is from Dr. Gary Nabel and Dr. Neil Perkins. The recombinant adenoviruses expressing Myo/V1 and β-galactosidase were constructed as follows. For AdMyo/V1/Adβgal, the respective expression plasmids pcDNA3-AM1.1-Myo/V1 and pcDNA3-βgal were incorporated into Ad5 adenovirus through allelic recombination. Recombinant adenoviruses were propagated, purified, and titered as previously reported (46).

**Cell Biology Techniques—**HeLa cells (ATCC—CCL2) were maintained in minimal essential medium. Cell fixation and indirect immunofluorescence studies were performed as previously described (47). Cytoplasmic and nuclear extracts were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce). Plasmid DNA transient transfection experiments were performed using FuGENE6 reagent (Roche). Luciferase assays were conducted with reagents from Promega Inc. The expression plasmid is from Dr. Gary Nabel and Dr. Neil Perkins. The adenovirus construct of interest was transfected into HeLa cells using FuGENE6 reagent (Roche). Luciferase assays were conducted with reagents from Promega Inc. The protein concentration was determined by BCA method using Pierce reagents.

HeLa cells were treated for 2 h with TNF (50 ng/ml) to induce NFkB. For superinduction of NFkB, cells were further treated with cycloheximide (10 μg/ml). After 2 h of treatment, cells were harvested and subcellular fractionation for cytoplasmic and nuclear extracts were carried out as previously described (48). Briefly, HeLa cells were harvested at 150 × g and Dounce-homogenized in a hypotonic lysis buffer (buffer A; Ref. 49), and nuclei were collected at 4300 × g. The nuclei were incubated with buffer C (49) and were used for GSA and Western blot analysis. After the removal of the nuclei, the supernatant was further centrifuged at 20,000 × g for isolating mitochondrial fractions. The remaining cytosolic supernatant was further concentrated by acetone precipitation. Identical amounts of cytosolic (40 μg), nuclear extract (30 μg), and mitochondrial proteins (35 μg) were fractionated on a 10% Tris-Tricine SDS-PAGE and processed for Myo/V1 ECL immunoblotting.

**RESULTS**

**Myo/V1 Is Localized in the Cytoplasm and Nucleus—**Indirect immunofluorescence studies were conducted to locate the native Myo/V1 protein in HeLa cells. Under basal conditions (Fig. 1A), Myo/V1 was predominantly observed in a wide area of the cytoplasm surrounding the nucleus. Further analysis with confocal microscopy revealed a more prominent and intense Myo/V1 signal in the nucleus with a lesser extent (spotted green fluorescence in Fig. 1C). Upon treatment with TNF for 1 h, Myo/V1 was found to cluster around the perinuclear region in the cytoplasm. Additionally, Myo/V1 was found to increase slightly within the nucleus (green FITC masking the blue DAPI nuclear staining, resulting in pale blue nucleus) suggesting migration of Myo/V1.
to the nucleus (Fig. 1B). Because TNF is known to reorganize the cytoskeleton (54–56), the present observation of increased perinuclear clustering and nuclear migration of Myo/V1 in TNF-treated HeLa cells (Fig. 1B) suggests that Myo/V1 might be associated with the cytoskeleton and its associated organelles and might participate in the signal transduction process from these locations.

**Myo/V1 Translocates to Nucleus during Sustained Induction of NFκB**—Cellular partitioning studies were performed to determine the location of Myo/V1 under the NFκB inducing conditions. Cells were treated with TNF or TNF plus CHX for 2 h. Cytoplasmic, mitochondrial, and nuclear extracts were prepared and immunoblotted for Myo/V1 protein (Fig. 2). Under basal conditions the majority of Myo/V1 was present in the cytoplasmic fraction (lane 5 in Fig. 2B) and only a small quantity in the nuclear fraction (lane 3 in Fig. 2B). After TNF stimulation for 2 h, Myo/V1 levels in the cytoplasmic fraction did not change significantly (data not shown). However, with TNF plus CHX stimulation, the levels of Myo/V1 reduced significantly in the cytoplasmic fraction compared with control (lanes 5 and 6 in Fig. 2B). Additionally, a simultaneous increase in the levels of Myo/V1 was observed in the nucleus (lane 4 in Fig. 2B), indicating that the translocated Myo/V1 migrated from cytoplasm. We did not observe any Myo/V1 in mitochondria or nuclei (lanes 1 and 2 in Fig. 2B). These data suggest that, under acute stress conditions, a major relocation of Myo/V1 occurred within the cytoplasm (Fig. 1B), following which Myo/V1 is translocated to the nucleus.

**Myo/V1 Physically Interacts with NFκB Proteins**—To confirm that Myo/V1 physically interacts with NFκB proteins, co-immunoprecipitation studies were conducted both in vitro and in vivo. Fig. 3A shows that, in vitro, p50, p65, and c-Rel proteins strongly interacted with Myo/V1 protein (lanes 2–4). To further confirm these physical interactions in vivo, HeLa cells were treated with diluent (lanes 8–10 in Fig. 3B), TNF plus CHX (lanes 5–7 in Fig. 3B), or phorbol ester (lanes 11–13 in Fig. 3C) for 2 h or infected with AdMyo/V1 for 12 h (lanes 14 and 15 in Fig. 3C), cellular extracts were prepared, and co-immunoprecipitation experiments were conducted. In control diluent-treated cells, Myo/V1 did not associate with any of the NFκB proteins (lanes 8–10 in Fig. 3B). However, in TNF plus CHX- or AdMyo/V1-treated cells, Myo/V1 predominantly associated with p65 protein (lane 7 in Fig. 3B and lane 15 in Fig. 3C) but not with p50 protein (lane 6 in Fig. 3B and lane 14 in Fig. 3C). In phorbol ester-treated HeLa cells (Fig. 3C), Myo/V1 co-immunoprecipitated with c-Rel and p65 protein (lanes 11 and 12 in Fig. 3C) compared with p50 protein (lane 13 in Fig. 3C).

**Myo/V1 Promotes the Formation of p50-p50 Homodimers from Monomeric p50 Proteins in Vitro**—Because our earlier studies indicated that Myo/V1 disrupts and induces the formation of new NFκB-DNA complexes (3) and can physically interact with NFκB proteins in vitro and in vivo (Fig. 3), we designed a detailed study to identify the role of Myo/V1 in altering the specific NFκB dimers in vitro. For this purpose, we initially studied the effect of Myo/V1 on monomeric NFκB proteins. NFκB proteins, p50, and p65 have low affinity to form homodimers among themselves, and a majority of the proteins remain monomeric without binding to κB DNA. However, when incubated together, they exhibit high affinity to its heterologous partner and they form heterodimers very easily. Purified recombinant p50 protein and truncated p65Δ protein (from Dr. Gaurishankar Ghosh) was used for these studies. First, monomeric p50 proteins were mixed at very low concentrations (2.5 ng in 50 μl; 2 nm) with Myo/V1 (0–100 ng) and the formation of p50-p50 homodimers were studied using gel-shift assay (lanes 1–5 in Fig. 4A). Similarly, monomeric p65Δ proteins were mixed at very low concentrations (0.5 μg in 50 μl; 7 μM) with Myo/V1 (0–100 ng), the formation of p65Δ-p65Δ homodimers
Because of the deletion of the carboxyl terminus of p65 protein, dimers is similar to that previously described (43, 51, 52). This observed subtle difference in the mobility between these p65 subunit of NF\(\kappa\)B heterodimers. Three myo/V1 actively promotes the formation of p50-p65 heterodimers. Close examination of the migratory patterns and the levels of these dimers (lanes 8 and 9 in Fig. 4C) reveals that, in the presence of p65 antibody, Myo/V1 promoted the formation of p50-p50 homodimers from the residual free monomeric p50 proteins, although at a reduced level. The splitting of the heterodimers as observed in lanes 2 and 3 did not occur in these reactions (lanes 8 and 9) because of the presence of p65 antibody. The NF\(\kappa\)B dimers that appear in lanes 7–9 are predominantly p50-p50 homodimers, as evidenced by its location.

To further confirm the chaperone functions of Myo/V1, we conducted another experiment (Fig. 4D), where the amount of p65\(\Delta\) was kept excess by 100-fold to that of p50 so that, in addition to the preformed p50-p65\(\Delta\) heterodimers, free p65\(\Delta\) proteins will be present in the reaction. The effect of Myo/V1 (lanes 1–3 in Fig. 4D), and the effect of NF\(\kappa\)B antibodies on these MYO/V1-shifted complexes were studied (lanes 6–8 and 10–12 in Fig. 4D). The rationale for this experiment is that, when the p50-p65 heterodimers are split by Myo/V1, there will be a competition among the released monomeric proteins to form either homodimers or heterodimers. With excess p65 protein in the reaction and because of its high affinity toward p50 protein to form heterodimers (43), there will be a continuous.
Fig. 4. Myo/V1 promotes the formation of p50-p50 homodimers from monomeric p50 proteins and converts NFκB p50-p65 heterodimers to p50-p50 and p65-p65 homodimers in vitro. A, GSA showing the effect of Myo/V1 on monomeric NFκB proteins. Increasing concentrations of Myo/V1 (0–100 ng in 25-ng increments) were added to p50 (lanes 1–5; 2.5 ng in 50 μl) and p65 proteins (lanes 6–10; 0.5 μg in 50 μl) separately, and κB-DNA binding activity was measured. Lanes 1 and 6 consist of p50-p50 and p65-p65α-p65α heterodimers, respectively, without Myo/V1. In lanes 2–5 and in lanes 7–10, increasing amounts of Myo/V1 (0–100 ng) were added. B, GSA showing the effect of Myo/V1 on preformed NFκB p50-p65α heterodimers. Increasing concentrations of Myo/V1 were added to preformed p50-p65α heterodimers, and κB-DNA binding activity was measured. p50 and truncated p65α protein were incubated to form p50-p65α heterodimers. To serve as a reference, preformed p50-p50 homodimers, p65-p65α heterodimers, and p65α-p65α homodimers are shown in lanes 1, 2, and 12. Increasing concentrations of Myo/V1 protein were added to preformed heterodimers in lanes 3–11. The subtle upward shift of the protein-DNA complex in lanes 3–5 is indicated by parallel arrows. Arrows in lanes 9–11 denote the freed p65α subunits forming p65α-p65α homodimers. C, GSA showing the effect of NFκB antibodies on Myo/V1-generated NFκB dimers. Increasing concentrations of Myo/V1 were added to preformed p50-p65α heterodimers (lanes 1–3), and p50 (lanes 5–7) and p65 antibodies (lanes 9–11) were added. ss, supershifted complex. D, GSA showing the effect of NFκB antibodies on Myo/V1-generated NFκB dimers with excess p65α protein. Increasing concentrations of Myo/V1 were added to preformed p50-p65α heterodimers with 100-fold excess p65α protein (lanes 1–3), and p50 (lanes 5–7) and p65 antibodies (lanes 9–11) were added. ss, supershifted complex. These results are representative of at least three different experiments.

formation of new p50-p65 heterodimers as the old heterodimers are being converted by Myo/V1. Thus, in this experiment Myo/V1-generated “intermediate products” can be identified. The p50-p65α heterodimers were pre-assembled at 1:100 ratios, and Myo/V1 was added at two (100 and 400 ng) concentrations (lanes 2 and 3 in Fig. 4D). These reactions were performed in triplicate for p50 and p65 antibody supershifts (lanes 5–7 and 9–11 in Fig. 4D). As expected (shown in Fig. 4D), both heterodimers and homodimers were formed when Myo/V1 was present at high concentrations with excess p65 (lane 3). The complete subtle upward shift observed in lane 3 of Fig. 4C was not similarly observed in lane 3 of Fig. 4D, strongly indicating the presence of both heterodimers and homodimers. The presence of both of these dimers is more clearly seen in lanes 7 and 11. Additionally, we also observed a simultaneous reduction in p65Δ-p65Δ homodimers (lanes 1–3) with the addition of Myo/V1. Because Myo/V1 does not have any direct effect on p65 per se (lanes 6–10 in Fig. 4A), the reduction in p65Δ-p65Δ homodimers in lanes 1–3 is a result of its incorporation into p50 protein in the reaction to form p50-p65Δ heterodimers. Furthermore, the absence of p50-p65Δ heterodimers in lanes 6 and 10 and the presence of these dimers in lanes 7 and 11 correlate with the reduction of p65Δ-p65Δ homodimers in lanes 7 and 11. Lanes 5–7 confirm that the Myo/V1-generated dimers have p50 proteins, as revealed by supershifted complexes. Because of excess p65, p50 antibody supershifts did not occur completely. Because the p65 antibody used in lanes 9–11 exhibits inhibitory activity on p65 proteins, the complete conversion of heterodimers to homodimers is affected. Thus, these results further confirm the chaperone functions of Myo/V1 in promoting NFκB homodimers and the formation of p50-p65Δ heterodimers is an indirect event promoted by the chaperone functions of Myo/V1 on p50.

Overexpression of Myo/V1 Changes the Ratio of Activated NFκB Dimers in Vivo Favor of p50-p50 Homodimers—To further confirm our in vitro results, in vivo studies were con-
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Fig. 5. Myo/V1 changes the ratio of NFκB dimers in vivo in favor of p50-p50 homodimers. A, GSA showing the effect of Myo/V1 on in vivo generated NFκB dimers. HeLa cells were infected with AdMyo/V1 or Advgal recombinant adenoviruses at a multiplicity of infection of 10. Twelve hours after infection, nuclear extracts were prepared and GSAs were conducted with three high affinity NFκB dimer-specific oligonucleotides (κB#seqB for p50-p50 homodimers; κB#u-iNOS for p65-p65 homodimers; IgGκB oligonucleotide for p50-p65 heterodimers) (lanes 1–6). Supershift experiments with NFκB κB50 and p65 antibodies were conducted to confirm the nature of the NFκB dimers (lanes 7–15). p50 supershift complexes are indicated by *, and p65 supershift complexes are indicated by arrows. B, quantitative comparison of NFκB dimers between Advgal and AdMyo/V1. The bar graph shows the fold change in the levels of individual NFκB dimers in relation to Advgal-infected HeLa cells. C, relative levels of NFκB dimers in Advgal- and AdMyo/V1-infected HeLa cells. These results are representative of six different experiments, conducted four times at a multiplicity of infection of 10 and twice at a multiplicity of infection of 50.

ducted to identify the role of Myo/V1 in NFκB homodimer generation. For this purpose, a strategy of adenovirus-mediated overexpression of Myo/V1 was chosen to exploit the inherent activation of NFκB heterodimers by recombinant adenoviral vectors (57–59). Thus, Myo/V1 overexpressing recombinant adenovirus is expected to alter the composition of activated NFκB dimers induced by its viral vector backbone (57–59). HeLa cells were infected with recombinant adenoviruses expressing Myo/V1 (AdMyo/V1) and β-galactosidase (Advgal), and nuclear extracts were prepared 12 h after infection. κB DNA binding reactions (Fig. 5A) were conducted with three high affinity NFκB dimer-specific oligos, and the levels of individual NFκB dimers were quantified (Fig. 5, B and C). Identical amounts of κB oligos (25,000 cpm) with similar specific activities were incubated with identical amounts of nuclear extracts, and the resulting NFκB-DNA complexes were fractionated on the same gel before autoradiography.

Results show that overexpression of AdMyo/V1 enhanced the generation of p50-p50 (lane 2 in Fig. 5A) and p65-p65 (lane 4 in Fig. 5A) homodimers compared with the control Advgal (lanes 1 and 3 in Fig. 5A). Interestingly, p50-p65 heterodimers are less abundant in AdMyo/V1 compared with the Advgal-overexpressing cells (lanes 5 and 6 in Fig. 5A). Thus, the observation of a simultaneous decrease in the levels of p50-p65 heterodimers and an increase in p50-p50 and p65-p65 homodimers suggests a conversion from heterodimers to homodimers (Fig. 5B). Moreover, an unequal quantitative shift (Fig. 5B) occurred between p50-p50 homodimers and p50-p65 heterodimers in Myo/V1-overexpressing cells. Although the levels of p50-p50 homodimers were elevated by 70%, the p50-p65 heterodimers declined by 26% (Fig. 5B). This observation suggests that Myo/V1, in addition to generating the p50-p50 homodimers from the p50-p65 heterodimeric substrates, might directly generate nascent p50-p50 homodimers from other in vivo substrates, further confirming our previous in vivo results (Fig. 4B). Furthermore, analysis of the relative levels of NFκB dimers (Fig. 5C) in AdMyo/V1-infected cells (p50-p65:p50-p50:p65-p65; 1.0:1.3:0.5) revealed that the levels of p50-p50 homodimers exceeded the levels of p50-p65 heterodimers compared with Advgal (1.0:0.55:0.2). Because p50-p50 homodimers possess transcriptionally repressive activity (17–22), the observed shifts in the NFκB dimer ratio in Myo/V1-overexpressing cells might have repressive effects on the NFκB-mediated transcription process.

Antibody supershift experiments and heterologous κB oligo chase experiments (data not shown) confirmed the nature of NFκB dimers (lanes 7–15 in Fig. 5A) binding to the κB oligos κB#SeqB, κB#Igκ, and κB#u-iNOS. However, the p50 antibody supershifting the NFκB complexes bound to κB#u-iNOS oligo (lane 11 in Fig. 5A) was unexpected. We believe this is because of “antibody-induced effect” rather than residual p50-p65 heterodimers binding to these κB oligos. The p50 antibody after binding to a p50-p65 heterodimer could change the affinity of the “supershifted heterodimeric ternary complex” now to recognize and bound to a otherwise p65-p65 homodimer specific oligo. Although antibodies used in gel-shift assays are generally described as “supershifting antibodies,” these antibodies change the affinity of homo- and heterodimers and thereby affect their binding to κB sites. Evidence of these artifacts is seen in lanes 12 and 15 where increased (lane 12) and decreased (lane 15) binding is observed after the antibody was added. Therefore, as described previously (44, 45) without the NFκB antibodies, κB#seqB and κB#u-iNOS oligos exhibit high affinity and exclusive specificity toward p50-p50 and p65-p65 homodimers. Additionally, we conducted heterologous κB oligo chase experiments (κB#u-iNOS-bound complexes chased with 100× cold κB#SeqB and κB#Igκ oligos), and the results revealed that Myo/V1-overexpressing HeLa cells still produced higher levels of p50-p50 and p65-p65 homodimers. Notably, additional chase experiments (κB#SeqB complexes chased with 100× cold κB#u-iNOS and κB#Igκ oligos; κB#Igκ complexes chased with 100× cold κB#u-iNOS oligos) were done, and the results were exactly the same as in Fig. 5A (data not shown).

Myo/V1 Represses NFκB-mediated Transcription Process—To identify the functional relevance of Myo/V1 with respect to NFκB dimers, we studied the role of Myo/V1 on NFκB-mediated transcription. HeLa cells were cotransfected with pBS-IκB-luc, pB5 pRSV-RelA, and pcDNA-AM1.1-Myo/V1 expression vector. Forty-eight hours after transfection, HeLa cells were harvested and lysed and luciferase enzyme activity was measured. The results (Fig. 6) show that overexpression of Myo/V1 significantly reduced the NFκB-mediated transcription by 42% (p < 0.05) on p565-mediated luciferase reporter activity. Because p50-p65 heterodimers are not available for
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Myo/V1 protein without the SRD, NLS masking, and AR domain-interacting ankyrin repeats and resembles a truncated form of IκB protein without the SRD, NLS masking, and PEST degradation domains (Fig. 7D). Results presented in this study indicate that, under basal conditions, cellular Myo/V1 predominantly present in the cytoplasm and probably associates with cytoskeletal structures (Fig. 1) similar to IκBα (61, 62). Additionally, low levels of Myo/V1 are also present in the nucleus (Fig. 1C) of unstimulated HeLa cells and in other cell types such as Jurkat T cells.

**DISCUSSION**

Myo/V1 was first identified as an up-regulated intracellular protein in pathophysiological conditions (1, 4–6) such as human heart failure, as well as during early organ developmental processes such as in postnatal mammalian cerebellum (2, 4, 8, 10). However, later it was identified to express in every mammalian organ and cell type. Moreover, Myo/V1 is an evolutionarily conserved protein from multicellular organisms to mammals (Fig. 8). Among mammals it shows 97% homology, and with invertebrates it exhibits 67% homology. Putative nuclear import and export signals as well as potential phosphorylation sites were mostly conserved between invertebrates and vertebrates (Fig. 8). Our three-dimensional structural alignment studies (Fig. 7C) further revealed that Myo/V1 possessed only Rel domain-interacting ankyrin repeats and resembles a truncated form of IκB protein without the SRD, NLS masking, and PEST degradation domains (Fig. 7D).

Results presented in this study indicate that, under basal conditions, cellular Myo/V1 predominantly present in the cytoplasm and probably associates with cytoskeletal structures (Fig. 1) similar to IκBα (61, 62). Additionally, low levels of Myo/V1 are also present in the nucleus (Fig. 1C) of unstimulated HeLa cells and in other cell types such as Jurkat T cells.
and rat neonatal cardiac myocytes. Our studies also demonstrated that, upon stimulation with TNF, Myo/V1 relocates within the cytoplasm (Fig. 1B) and further translocates to the nucleus during sustained induction of NF\(^\kappa\)B (Figs. 1B and 2B). Because Myo/V1 possesses both nuclear import and export signals (Fig. 8), it is expected that Myo/V1 might shuttle between cytoplasm and nucleus similar to I\(^\kappa\)B family of proteins (32–36). Co-immunoprecipitation studies showed that, although Myo/V1 interacts with all NF\(^\kappa\)B proteins in vitro, it remains physically associated only with p65 and c-Rel proteins in vivo (Fig. 3). The differences between in vitro and in vivo results might also reflect the potential function of Myo/V1 protein. For example, Myo/V1 may interact dynamically with p50 proteins in vivo in a transient fashion to mediate its zipper chaperone function; however, it remains physically associated with p65 or its homologue c-Rel protein to prevent them from reassociating with p50.

In vitro NF\(^\kappa\)B interaction studies indicated that Myo/V1 can generate p50-p50 homodimers from monomeric p50 proteins and can convert the preformed p50-p65 heterodimers into p50-p50 and p65-p65 homodimers (Figs. 4 and 9A). Compared with control Ad\(^\kappa\)H9252 gal, overexpression of AdMyo/V1 resulted in an increase in both p50-p50 and p65-p65 homodimers exceeding the levels of p50-p65 heterodimers (Fig. 5). A simultaneous decrease in the levels of p50-p65 heterodimers was also observed, suggesting a conversion from transactivating heterodimers to transcriptionally repressive homodimers (17–25). However, the observation of disproportionate increase in the levels of p50-p50 homodimers compared with the decreased levels of heterodimers (70% increase versus 26% decrease) raises the possibility that Myo/V1 might also generate p50-p50 homodimers from other inactive NF\(^\kappa\)B substrates. Because a majority of the Myo/V1 is localized in the cytoplasm, it could very well be involved in the activation of p50-p50 homodimers by acting on p50-p105 substrates (63) in vivo (Fig. 9B).

Alternatively, Myo/V1 could assemble the p50-p50 homodimers from free monomeric p50 proteins in the cytoplasm similar to the conditions mentioned in Fig. 4A. Additionally, Myo/V1 could act on any p50 subunit containing complexes (p50-p65, p50-c-Rel, p50-RelB, and “p50-non-NF\(^\kappa\)B” dimers) in the nucleus and further generate the transcriptionally repressive p50-p50 homodimers (Fig. 9B). In accordance with these observations, overexpression of Myo/V1 also resulted in significantly reduced NF\(^\kappa\)B-mediated luciferase reporter gene expression (Fig. 6), which is probably a result of the generation of p50-p50 homodimers.

Although we observed an increased level of p65-p65 homodimers during AdMyo/V1 overexpression (Fig. 5A), and Myo/V1 being physically associated with p65 and its homo-
logsues (Fig. 3), in vitro interaction experiments (Fig. 4A) do not suggest an active role for Myo/V1 in the generation of p50-p65 heterodimers. Moreover, in overexpression of Myo/V1 in HeLa cells through transient transfection methods (Fig. 6), where “active p50-p65 heterodimers” are not available for Myo/V1 to act upon, we only observed an inhibition of k-B driven luciferase reporter activity. If Myo/V1 would have actively promoted the generation of p50-p65 homodimers in these cells, we should have observed an increased k-B-luciferase reporter activity during Myo/V1 overexpression. Because Myo/V1 does not retain or sequester p65 in the cytoplasm under basal conditions (Fig. 6), we identified a strong temporal relationship between the conversion of p50-p50 homodimers by Myo/V1, as observed in our in vitro and in vivo experiments (Figs. 4 and 5). Therefore, we believe the increased generation of p50-p50 homodimers during AdMyo/V1 expression is an indirect effect of the conversion of p50-p50 heterodimers by Myo/V1 and the physical association of Myo/V1 with p65 is probably to prevent the p65 from reassOCIating with p50 (Fig. 9A).

Even though our studies indicate that Myo/V1 could favor the active generation of NFkB homodimers in HeLa cells, it is possible that, under certain scenarios (similar to conditions mentioned in Fig. 4D), Myo/V1 could indirectly induce the formation of NFkB heterodimers in other cell types. For example, Myo/V1 could potentially act on inactive p50-p65-IkBa substrate complexes in the cytoplasm and generate active NFkB heterodimers in immune cells. Because Myo/V1 has been identified as "a severe combined immunodeficiency complementing gene" (GenBank accession no. D78188), and because this protein is also found in the nuclear extracts of phorbol ester-treated Jurkat T cells, the potential role of Myo/V1 in the activation of other NFkB dimers is highly likely.

The data presented in this study were further strengthened when, from previously published independent studies (2, 64–66), we identified a strong temporal relationship between the intracellular levels of Myo/V1 and NFkB activation in vitro in mammalian organs. Myo/V1 was found to occur at elevated levels in failing human hearts (1, 4–6) and found to be transiently elevated in postnatal mammalian cerebellum (2, 7). Interestingly, NFkB dimers were also found to be activated in failing human hearts (67) as well as in developing postnatal cerebellum (64–66) during this period. Specifically, in postnatal cerebellum, the intracellular level of Myo/V1 protein was found to rise at the 2nd day, peak at the 7th day, and rapidly decrease by the 12th day (2). Similarly, independent studies have reported the transient activation of NFkB dimers begin to occur around postnatal day 2, peaking at the 7th day, and declining by 12th day in rat (64, 65) and mouse cerebellum (66). This strong temporal observation that the intracellular levels of Myo/V1 being elevated exactly at the same time during NFkB dimer activation and its decline during the disappearance of NFkB dimers in postnatal cerebellum further highlights the important potential role of Myo/V1 even in initial NFkB activation. The earlier observation of disproportionate increase in the levels of p50-p50 homodimers with the decreased levels of heterodimers in AdMyo/V1-infected cells (Fig. 5) also raises the possibility that Myo/V1 might be involved in the initial activation of p50-p50 homodimers in vivo (Fig. 9B).

Therefore, the results presented in this study strongly suggest that not only Myo/V1 is involved in converting heterodimers into homodimers during sustained NFkB activation, it could potentially be involved in the initial activation of NFkB dimers. The recent identification that entire NFkB machinery (NIK, IKKs, IkBa, ubiquitination, and proteasome) is shuttling (68, 69) between cytoplasm and the nucleus of resting mammalian cells (32–35) and the observation of NFkB activation without Ik degradation (70, 71) changes the central tenet of NFkB signaling mechanism. In this changed scenario, several alternate Ik-independent pathways (70–72) have been involved in such pathways.

The identification of Myo/V1 as a NFkB zipper chaperone (Fig. 9A) will shift the NFkB dimer ratio from transcriptionally active heterodimers to transcriptionally repressive homo- dimers and is also highly significant. In fact, in many biological scenarios like B lymphocyte cell differentiation (40–42), lipopolysaccharide tolerance (73), T-lymphocyte anergy (74), and chronic TNF exposure to mouse myocardium (75), the activated NFkB dimers are known to change both quantitatively and qualitatively during these events. For example, during B lymphocyte cell differentiation (40–42), dynamic exchange of NFkB subunits are known to occur among the activated NFkB dimers resulting in altered gene expression appropriate for the stage of differentiation. During endotoxin tolerance in monocytes (73), T-lymphocyte anergy (74), and chronic TNF exposure to mouse myocardium (75), the levels of p50-p50 homodimers are known to increase during late stages of these events, suggesting a dynamic qualitative and quantitative change in the activated NFkB dimers. Moreover, in human sepsis, large amounts of p50-p50 homodimers exceeding the levels of p50-p65 heterodimers were observed in monocytes of non-survivors compared with the survivors, further suggesting an important role for NFkB dimer ratio in the pathophysiology of sepsis (76). Furthermore, the NFkB dimer ratio has been shown to determine the ultimate level of target gene expression (18, 22). NFkB-mediated gene transcription has been proposed in models of myocardial diseases (3, 77, 78). Taken together with the previous finding that elevated levels of Myo/V1 are found in failing mammalian hearts (5, 6), this study collectively suggests that Myo/V1 might play a significant NFkB regulatory role at the transcriptional level during chronic pathophysiological conditions such as human heart failure.

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