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LXXLL motifs and AF-2 domain mediate SHP (*NR0B2*) homodimerization and DAX1(*NR0B1*)-DAX1A heterodimerization

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Abstract

Small heterodimer partner (SHP; *NR0B2*) is an unusual orphan member of the nuclear receptor superfamily that functions as a corepressor of other nuclear receptors through heterodimeric interactions. Mutations in SHP are associated with mild obesity and insulin resistance. The protein domain structure of SHP is similar to Dosage-sensitive sex reversal adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1 (DAX1; *NR0B1*). Mutations in DAX1 cause AHC with associated hypogonadotropic hypogonadism. DAX1A is an alternatively spliced isoform of DAX1 that lacks the last 80 amino acids of the DAX1 C-terminal repressor domain and is replaced by a novel 10-amino acid motif. We have previously shown homodimerization of SHP and DAX1 individually, heterodimerization of DAX1 with SHP, and heterodimerization of DAX1 with DAX1A. In these studies, we investigated the domains and residues of SHP involved in SHP homodimerization and DAX1-SHP heterodimerization and also further characterized DAX1-DAX1 homodimerization and DAX1-DAX1A heterodimerization. We showed involvement of the SHP LXXLL motifs and AF-2 domain in SHP homodimerization and DAX1-SHP heterodimerization. We demonstrated redundancy of the LXXLL motifs in DAX1 homodimerization. While DAX1A subcellular localization is mostly cytoplasmic, DAX1-DAX1A heterodimers existed in the nucleus, suggesting differential functions for DAX1A in each compartment. We showed that the AF-2 domain of DAX1 is involved in DAX1-DAX1A heterodimerization. These results indicate that NR0B family members use similar mechanisms for homodimerization as well as heterodimerization. These resemble coactivator-receptor interactions that may have potential functional consequences for molecular mechanisms of the NR0B family.

Keywords

SHP; DAX1; DAX1A; Nuclear Receptor; Homodimerization

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Introduction

SHP (small heterodimer partner; *NR0B2*) and DAX1 (Dosage-sensitive sex reversal adrenal hypoplasia congenita (AHC) critical region on the X chromosome, gene 1; *NR0B1*) are members of the unusual NR0B family of orphan nuclear receptors. SHP and DAX1 both contain a carboxy-terminal domain (CTD) homologous to other nuclear receptors including the presence of the activation function (AF)-2 domain. What makes these receptors unusual, however, is the lack of the canonical DNA-binding, modulator, and hinge regions [1-3]. The amino-terminal domains (NTD) of SHP and DAX1 instead contain one or more repeats of a novel 65-70 amino acid alanine/glycine-rich motif that has no homology to any known protein. SHP has a short NTD with one 70 residue repeat, and DAX1 has a longer NTD containing 3.5 repeats [1,4]. An alternatively spliced isoform of DAX1, DAX1A, has been identified and contains an NTD similar to DAX1, but the CTD of DAX1A lacks the last 80 amino acids of the DAX1 CTD including theAF-2 domain, that is replaced by a novel 10-amino acid motif [5,6]. No alternatively spliced isoforms have been identified for SHP.

SHP is expressed in a wide spectrum of tissues as SHP mRNA has been detected in the liver (fetal and adult), pancreas, heart, adrenal gland (fetal and adult), spleen, testis, ovary, adipose tissue, stomach, small intestine, and brain [1,7-11]. Roles for SHP in the liver, pancreas, and adipose tissue have been the most extensively characterized as SHP has been shown to be a key player in lipid and glucose metabolism [12]. This role is further supported by the fact that mutations in *NR0B2* are associated with mild obesity, high birth weight, and insulin resistance in certain populations [13,14].

DAX1 is expressed throughout the hypothalamic-pitutary-adrenal-gonadal axis, and mutations in DAX1 cause adrenal hypoplasia congenita (AHC) with associated hypogonadotropic hypogonadism, suggesting a critical role for DAX1 in the development and adult function of this axis [4,15-21]. DAX1 may also have a role in bone cell development as well as early embryonic development [22-24]. DAX1A has a wider tissue expression profile than DAX1, as it is expressed in the adrenal gland, brain, ovary, pancreas, and testis, but the function of DAX1A in these tissues is unclear [5,6].

Nuclear receptors typically function as activators of gene expression by binding to DNA response elements on target genes followed by the recruitment of coactivator proteins [2,3]. This classical receptor activation function has not been shown for SHP or DAX1. Rather, SHP and DAX1 have been shown to repress the transcriptional transactivation of many members of the nuclear receptor superfamily [12,25]. Coactivator proteins typically bind the AF-2 domain of nuclear receptors via their LXXLL motifs, or NR-boxes, to facilitate transcriptional activation. SHP contains two and DAX1 contains three LXXLL motifs and are thus considered LXXLL-containing co-repressors. Both SHP and DAX1 repress the action of many nuclear receptors through a two-step mechanism of coactivator competition followed by corepressor recruitment. SHP and DAX1 bind to the AF-2 domain of other nuclear receptors through their LXXLL motifs, which is thought to result in competition for coactivator binding. This is followed by active repression through a silencing function in the CTD of these receptors that is thought to involve corepressor recruitment and histone deacetylases [12,25].

A repressor function for DAX1A has not been shown, as it lacks the capacity to repress SF1 transactivation, but is instead thought to antagonize the action of DAX1. Hossain et al. [6] showed that DAX1A interacts with SF1, and therefore suggest that DAX1A antagonizes DAX1 repression by competing with DAX1 for binding to SF1. We demonstrated the existence of DAX1-DAX1A heterodimers [26] and proposed an alternative mechanism whereby DAX1A interacts with DAX1 and prevents DAX1 from interacting with and/or repressing SF1.

Nuclear receptors function in one or more dimeric combination for functional complexity [3, 27]. A function for DAX1 and SHP independent of other nuclear receptors has been suggested by our recent work that demonstrates that DAX1 and SHP each form homodimers individually, as well as DAX1-SHP heterodimers [26]. DAX1 homodimerizes in the nucleus and cytoplasm, and SHP homodimers and DAX1-SHP heterodimers exist in the nucleus. DAX1 and SHP homodimers dissociate in the presence of a nuclear receptor target, i.e. ligand-activated ERα, suggesting that these homodimers act as a holding reservoir in the absence of heterodimeric partners. DAX1-DAX1A heterodimers also form. These different dimeric combinations suggest complexity in molecular mechanisms for members of the NR0B family.

Nuclear receptors typically form parallel homo- or heterodimers through contacts in specific portions of the DBD and LBD [2,27]. Our previous studies show that DAX1 homodimerizes differently from other members of the nuclear receptor superfamily [26]. We provide evidence for the formation of an anti-parallel homodimer through an interaction between the LXXLL motifs of the NTD of DAX1 with the AF-2 domain in its CTD, which is similar to the heterodimeric interactions between DAX1 and its nuclear receptor targets. We also previously demonstrated the involvement of these DAX1 residues in DAX1-SHP heterodimerization. The residues of SHP involved in its dimeric interactions, as well as the domains and residues involved in DAX1-DAX1A heterodimerization have not been characterized.

In this study, we show the involvement of the SHP LXXLL motifs and AF-2 domain in SHP homodimerization and heterodimerization with DAX1. We further demonstrate redundancy of the LXXLL motifs in DAX1 homodimerization. We characterize the subcellular localization of DAX1A as well as the DAX1-DAX1A heterodimers and show that DAX1A has a mostly cytoplasmic localization, but DAX1-DAX1A heterodimers exist mostly in the nucleus. We also show that DAX1-DAX1A heterodimerization involves an LXXLL-AF-2 interaction. These results show that NR0B family members utilize similar mechanisms for homodimerization as well as heterodimerization with each other and with other nuclear receptors, and also demonstrate that NR0B interaction mechanisms are distinct from dimeric interactions of the majority of members of the nuclear receptor superfamily.

Materials and Methods

Plasmids

pShuttle-Flag3 [28] was used to express FLAG-tagged proteins and pCMVTag3C (Stratagene, La Jolla, CA) was used to express myc-tagged proteins. FLAG- and myc-tagged DAX1, DAX1-N, DAX1-C, SHP, and DAX1A constructs have been described [26]. Mutations were generated using the QuikChange Site-Directed Mutagenesis System (Stratagene) using PCR primers containing the mutations indicated the figures. Mutations were verified by sequencing.

Mammalian Cell Culture and Transfections—Human embryonic kidney 293 (HEK293) cells (BD Biosciences, San Jose, CA) were cultured in DMEM containing 10% Fetal Bovine Serum (FBS) and antibiotics at 37°C in 5% CO_2. Transfections were performed in 6-well plates. Cells were plated at 70-80% confluence 24h prior to transfection. Cells were transfected with appropriate expression plasmids (as indicated in figure legends) with quantities according to the manufacturer's recommendations using Lipofectamine2000 (Invitrogen, San Diego, CA). Whole cell extracts were prepared 36-40h post-transfection in RIPA Lysis Buffer (0.05M TrisHCl pH 7.4, 0.15M NaCl, 1% NP40, 1mM EDTA) in the presence of protease inhibitors (Halt Protease Inhibitor Cocktail EDTA-free, Pierce Biotechnology, Rockford, IL). Nuclear and cytoplasmic fractions were prepared using the NE-PER Nuclear and Cytoplasmic Extraction System (Pierce). Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA). Extracts and fractions were then analyzed for protein expression by western blotting or used in co-IP assays.

Western Blotting—Whole cell extracts and nuclear and cytoplasmic fractions were resolved by 10% SDS-PAGE (DAX1 and DAX1A detection) or 13% SDS-PAGE (SHP detection), transferred to polyvinylidine difluoride (PVDF) membrane, and subjected to western analysis using Anti-FLAG M2 Peroxidase Conjugate (Sigma-Aldrich, St. Louis, MO), Anti-c-myc-HRP (Clone 9E10, Santa Cruz Biotechnology), Anti-Hsp90 (clone AC88, Stressgen Biotech Corp.,Victoria, British Columbia, Canada), or Anti-mouse IgG-HRP (Stressgen) as indicated in the figures. Membranes were visualized with ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Co-IP Assays in Mammalian Cells—HEK293 whole cell extracts (1-2mg) or nuclear/cytoplasmic fractions (800μg) were incubated with 20μl Anti-FLAG-M2 affinity gel (Sigma) overnight at 4°C. The resin was then washed three times with PBS and bound proteins were eluted by boiling in 2x Laemmli buffer for 5min. 25% of the IP reaction was resolved by SDS-PAGE. Proteins were transferred to PVDF membranes and analyzed by Western blotting. Signal quantitation was performed by laser densitometry (Molecular Dynamics) with analysis using ImageQuant software (Amersham) on three independent experiments.

Results

LXXLL motifs and AF-2 domain are involved in SHP homodimerization

Since DAX1 homodimerization involved the LXXLL motifs and AF-2 domain [26], we hypothesized that SHP could homodimerize via a similar mechanism. SHP contains two LXXLL motifs, one in its NTD (residues 21-25), and one in its CTD (residues 118-122), and the AF-2 domain is located at residues 249-254 (Figure 1A). To study the involvement of these residues in SHP homodimerization, we used differentially epitope tagged proteins (FLAG and myc), and created a triple mutant, where both LXXLL motifs as well as the AF-2 were mutated (3mut:SHP) (Figure 1A), and compared binding of this mutant SHP with wildtype SHP. The last two highly conserved hydrophobic residues of each LXXLL motif were mutated to alanines, similar to previous studies [26,29], and the conserved negatively charged aspartic acid residue of the AF-2 domain, which forms part of the charge clamp [2,27], was mutated to arginine.

Co-IP assays were performed using whole-cell extracts of human embryonic kidney (HEK) 293 cells. Constructs expressing FLAG-SHP were cotransfected with either wildtype myc-SHP or triple mutant myc-SHP (myc-3mut:SHP) into HEK293 cells. Western blot analysis of whole-cell extracts verified equal protein expression across all samples (data not shown). Co-IP using a FLAG antibody showed an 83% decrease in the amount of myc-3mut:SHP pulled down with FLAG-SHP compared with wildtype myc-SHP (Figure 1B). These results indicate that the LXXLL motifs and AF-2 domain are involved in SHP homodimerization, and are consistent with the formation of an antiparallel SHP homodimer, similar to DAX1 homodimerization [26].

LXXLL motifs and AF-2 domain of SHP are involved in DAX1-SHP heterodimerization

DAX1 uses similar motifs and residues for its homodimerization as well as its heterodimerization with SHP [26]. We therefore hypothesized that the motifs and domains involved in SHP homodimerization are also involved in heterodimerization with DAX1.

To demonstrate the involvement of the SHP LXXLL motifs in heterodimerization with DAX1, we introduced point mutations of the last two highly conserved hydrophobic residues of both SHP LXXLL motifs (m12:SHP) and tested the effects of this mutation on binding to the DAX1 C-terminus (DAX1-C), which contains the DAX1 AF-2 domain (Figure 2A). Co-IP assays were performed on whole-cell extracts of HEK293 cells transfected with FLAG-DAX1-C and

either wildtype myc-SHP or mutant myc-m12:SHP. Western blot analysis confirmed equal protein expression across all samples (data not shown). Co-IP using a FLAG antibody showed an 82% decrease in the amount of myc-m12:SHP pulled down with FLAG-DAX1-C compared to wildtype myc-SHP (Figure 2B). These results indicate that the LXXLL motifs of SHP are involved in its heterodimerization with DAX1.

To determine the involvement of the SHP AF-2 domain in DAX1-SHP heterodimerization, we mutated the conserved negatively charged charge clamp residue of the SHP AF-2 domain (mAF-2:SHP) and tested the effects of this mutation on binding to the DAX1 N-terminus (DAX1-N), which contains the DAX1 LXXLL motifs (Figure 2A). Constructs expressing FLAG-DAX1-N and either wildtype myc-SHP or mutant myc-mAF-2:SHP were transfected into HEK293 cells. Western blot analysis confirmed equal protein expression (data not shown). Co-IP with a FLAG antibody showed a 55% decrease in the amount of myc-mAF-2:SHP pulled down with FLAG-DAX1-N compared with wildtype myc-SHP (Figure 2C). These results demonstrate involvement of the SHP AF-2 domain in DAX1-SHP heterodimerization.

Redundancy of LXXLL motifs in DAX1 homodimerization

DAX1 contains three LXXLL motifs in its NTD (Figure 3A), and SHP contains two LXXLL motifs, one in the NTD and one in the CTD (Figure 1A). We have shown that mutation of all LXXLL motifs reduces DAX1 homodimerization [26], and our present studies suggest that this is also the case for SHP. It is possible that one or more of these LXXLL motifs could be preferentially involved in these homodimeric interactions. We chose to use DAX1 to study the contribution of each LXXLL motif to homodimerization because DAX1 is a larger protein and is thus conducive for isolation of the LXXLL motifs using the DAX1 NTD. Constructs expressing wildtype FLAG-DAX1-N or FLAG-DAX1-N containing mutations in either the first (m1:DAX1-N), second (m2:DAX1-N), third (m3:DAX1-N), or all three LXXLL motifs (m123:DAX1-N) were cotransfected with wildtype myc-DAX1 into HEK293 cells (Figure 3A). Equal protein expression was confirmed by Western analysis (data not shown). Co-IP with a FLAG antibody showed that while mutation of all three LXXLL motifs resulted in an 80% decrease in the amount of myc-DAX1 pulled down with FLAG-m123:DAX1-N compared with wildtype, similar to our previous results [26], mutation of each LXXLL motif individually did not significantly impair this interaction (Figure 3B). These results suggest redundancy of the LXXLL motifs in DAX1 homodimerization and and we would speculate that there may be redundancy of these motifs in SHP homodimerization.

DAX1A localization is mostly cytoplasmic, but DAX1-DAX1A heterodimers exist in the nucleus

DAX1A is an alternatively spliced isoform of DAX1 that lacks the last 80 amino acids of the DAX1 CTD, and thus lacks the AF-2 domain [5,6]. Previous subcellular localization studies show that DAX1 localizes to both the nucleus and cytoplasm, with higher levels in the nucleus [26,30,31]. The subcellular localization of DAX1A has not been studied. Nuclear and cytoplasmic fractions were prepared from HEK293 cells transfected with either myc-DAX1A alone, or with FLAG-DAX1 and myc-DAX1A. Western blot analysis showed that hsp90 and endogenous c-myc localized to the cytoplasm and nucleus, respectively, indicating a relatively clean fractionation (Figure 4A). FLAG-DAX1 localized to the nucleus and cytoplasm (not shown). The localization of DAX1A was primarily cytoplasmic, with low levels in the nucleus, and this localization was not significantly altered in the presence of DAX1 (Figure 4A).

We have demonstrated previously that DAX1A does not homodimerize, but rather preferentially forms heterodimers with DAX1 [26]. We have shown that DAX1 homodimers exist in both the nucleus and cytoplasm, and that SHP homodimers and DAX1-SHP heterodimers exist in the nucleus [26]. The subcellular localization of the DAX1-DAX1A

heterodimer has not been studied and may provide insight regarding its function. The localization of the DAX1-DAX1A heterodimer was investigated by Co-IP of nuclear and cytoplasmic fractions of HEK293 cells co-transfected with FLAG-DAX1 and myc-DAX1A (Figure 4B). Co-IP with a FLAG antibody followed by western blot with a myc antibody showed that myc-DAX1A was immunoprecipitated with FLAG-DAX1 in the nuclear compartment, indicating that though DAX1A localized mostly to the cytoplasm, the DAX1-DAX1A heterodimers exist in the nucleus.

AF-2 domain of DAX1 is involved in DAX1-DAX1A heterodimerization

To determine the domains of DAX1 involved in heterodimerization with DAX1A, FLAG epitope-tagged constructs expressing full length DAX1, DAX1-N, or DAX1-C were cotransfected along with myc-DAX1A (Figure 5A) into HEK293 cells. Western analysis confirmed equal protein expression (data not shown). Co-IP with a FLAG antibody showed that myc-DAX1A was significantly pulled down with full length DAX1 as well as DAX1-C, but not with DAX1-N, indicating that the C-terminal half of DAX1 interacts with DAX1A (Figure 5B).

Since DAX1 homodimerization as well as heterodimerization with SHP and with other nuclear receptors involves an LXXLL/AF-2 interaction, it is possible that the AF-2 domain of DAX1 could interact with the LXXLL motifs of DAX1A. To determine if the AF-2 domain of DAX1 is involved in the interaction with DAX1A, constructs expressing either wildtype FLAG-DAX1-C or FLAG-DAX1-C containing a mutant AF-2 domain (FLAG-mAF-2:DAX1-C) (Figure 6A) were cotransfected with myc-DAX1A into HEK293 cells. Equal protein expression was confirmed by Western analysis (data not shown). Co-IP with a FLAG antibody showed a 72% decrease in the amount of myc-DAX1A pulled down with FLAG-mAF-2:DAX1-C compared with wildtype FLAG-DAX1-C (Figure 6B). These results demonstrate that the AF-2 domain of DAX1 is involved in DAX1-DAX1A heterodimerization.

Discussion

NR0B family members form antiparallel homo- and heterodimers

SHP and DAX1 form heterodimers with their nuclear receptor targets, i.e. ERα, through an interaction between their LXXLL motifs and the AF-2 domain of the target nuclear receptor [11,25,29,32,33]. We previously demonstrated a decrease in DAX1 or SHP homodimerization in the presence of increasing amounts of ERα, suggesting a competition for similar binding surfaces and subsequently showed the involvement of the LXXLL motifs and AF-2 domain in the formation of antiparallel DAX1 homodimers [26]. Here we demonstrated that the LXXLL motifs and AF-2 domain are also involved in SHP homodimerization. These results support the formation of antiparallel SHP homodimers. We also demonstrated that these residues in SHP are involved in DAX1-SHP heterodimerization and that the LXXLL motifs of DAX1 interact with the AF-2 domain of SHP, and vice versa, consistent with the formation of an antiparallel heterodimer. These interactions confirm a new interactional paradigm for this unusual family of nuclear receptors and provide additional evidence that the unusual members of the NR0B nuclear receptor family not only have domain structures different from other nuclear receptors, but also exhibit dimerization mechanisms different from most superfamily members. This interaction most resembles a receptor-coactivator interaction and could imply an unknown functional consequence.

The functional significance of three LXXLL motifs in DAX1 and two LXXLL motifs in SHP is unclear. Suzuki et al. [29] showed through a mammalian-two-hybrid analysis, that while all three motifs were capable of interacting with SF1 and ERα, the first and third motifs of DAX1 appeared to interact more strongly with SF1 and the third appeared to interact the strongest

with ERα. Both LXXLL motifs of SHP appear to be involved in repression and interaction with ERα [32], but only the second was functionally relevant for interaction with LRH-1 [34] and GR [35]. In our results shown here, while mutation of all three LXXLL motifs impaired DAX1 homodimerization, mutation of each LXXLL motif individually did not significantly impair homodimerization, indicating that all three motifs may be capable of interacting with the DAX1 AF-2 and that there is some redundancy in function. Our results strongly suggest that one or both of the SHP LXXLL motifs could be involved in SHP homodimerization. Mutagenesis studies by Johansson et al. [32] showed that both SHP LXXLL motifs were involved in the interaction with ERs, but structural studies by Li et al. [34] showed that only the second motif was involved in the interaction with LRH-1. It is possible that these motifs in SHP could be redundant in function for homodimer formation, and could exhibit target specificities depending on whether it is forming heterodimers with DAX1, or heterodimers with known repression targets.

While mutation of the LXXLL motifs of SHP nearly abolished interaction with the DAX1 CTD, as was observed with mutation of the DAX1 LXXLL motifs [26], mutation of the AF-2 domain of SHP did not completely eliminate heterodimerization with DAX1. These observations suggest the existence of additional SHP residues that may be involved in stabilizing the interaction with the DAX1 NTD.

DAX1A may have unique functions in both the cytoplasm and nucleus

DAX1 localizes to both the nucleus and cytoplasm, but the subcellular localization of DAX1A has not been previously studied. We observed DAX1A localizing primarily to the cytoplasm, with small levels in the nucleus. DAX1 did not significantly affect this localization. DAX1A lacks the last 80 amino acids of the DAX1 CTD and therefore does not contain the AF-2 domain [5,6]. Mutations in DAX1 that either delete or mutate key residues in the AF-2 domain cause a shift in DAX1 localization almost entirely to the cytoplasmic compartment [31,36,37], indicating that the AF-2 domain of DAX1 is involved nuclear localization. Our observation of a mostly cytoplasmic localization for DAX1A is consistent with these reports, and not only provides further evidence for the involvement of the AF-2 domain for proper DAX1 localization, but also points to a potential novel function for DAX1A in the cytoplasm.

Since DAX1 is also present in the cytoplasm, we hypothesized that DAX1-DAX1A heterodimers would exist in the cytoplasm. However, we did not observe significant heterodimerization of DAX1 with DAX1A in the cytoplasm. Rather, DAX1-DAX1A heterodimers existed mostly in the nucleus. This suggests that DAX1A could have differential functions in each compartment. DAX1A could have a specific function in the cytoplasm as a monomer, and nuclear DAX1A could be involved in antagonizing repression by DAX1 through heterodimerization.

DAX1A antagonizes DAX1-mediated repression of SF1, presumably by DAX1A competing for binding to SF1 [6]. We previously demonstrated the existence of DAX1-DAX1A heterodimers [26]. DAX1 binds to the AF-2 domain of SF1 through its LXXLL motifs [29], and it is very likely that DAX1A interacts with SF1 in a similar fashion. We have shown in these studies that DAX1 heterodimerizes with DAX1A through the same LXXLL/AF-2 interaction. This was supported by our observation that the DAX1 CTD interacted with DAX1A while the DAX1 NTD did not. DAX1A does not have an AF-2 domain, and therefore the LXXLL motifs of the DAX1 NTD could not interact with DAX1A. Mutation of the AF-2 domain of the DAX1 CTD decreased interaction with DAX1A confirming the involvement of this domain in this interaction and further supporting a mechanism of DAX1-DAX1A heterodimerization that is analogous to DAX1 homodimerization. The AF-2 domain of DAX1 very likely interacts with the LXXLL motifs of DAX1A. DAX1A has three LXXLL motifs identical to DAX1, and while the motifs exhibited redundancy in DAX1 homodimerization,

one or more of these motifs could be preferentially involved in heterodimerization with DAX1A.

Our observation that the LXXLL motifs of DAX1A interact with the AF-2 domain of DAX1 provides insights as to how DAX1A could antagonize the repressor activity of DAX1. DAX1 interacts through its LXXLL motifs with the AF-2 domain of SF1 [29]. DAX1A could interact with SF1, preventing DAX1 binding to SF1 as was proposed by Hossain et. al. [6]. Alternatively, DAX1 could interact with SF1 through its LXXLL motifs to repress SF1 transactivation, and DAX1A could bind to the DAX1 AF-2 domain, forming a trimeric complex, potentially interfering with the repressor function of the DAX1 CTD.

Homodimerization of DAX1 and SHP as well as heterodimerization of DAX1 with SHP and also with DAX1A adds additional complexity in the mechanisms of action of these nuclear receptors since it points to a function distinct from their known roles as transcriptional repressors. The homo- and heterodimerization mechanisms for these receptors are distinct from most members of the nuclear receptor superfamily, most resembling a coactivator-receptor interaction. It is possible that such an interaction may have unknown functional consequences for the molecular mechanisms of members of this unusual NR0B family of nuclear receptors.

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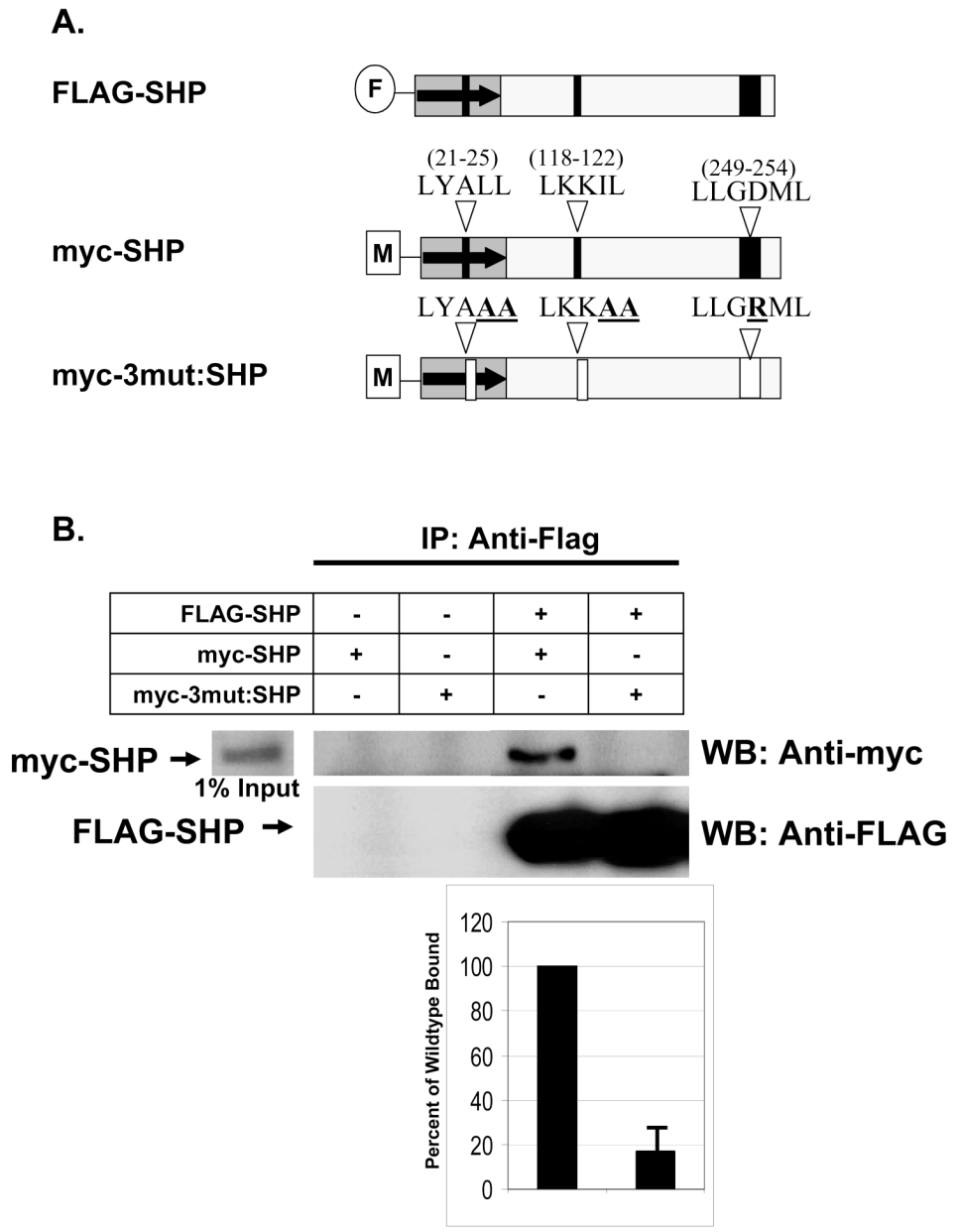


Figure 1. LXXLL motifs and AF-2 domain are involved in SHP homodimerization

(A) Schematic of FLAG- (F) and myc- (M) SHP constructs used in Co-IP assays. LXXLL and AF-2 residue numbers are in parentheses. Point mutations are in bold and underlined. Black and white lines represent wildtype and mutant LXXLL motifs, respectively. Black and white boxes represent wildtype and mutant AF-2 domain, respectively. (B) Co-IP assay. HEK293 cells were transfected with the indicated combinations of constructs expressing FLAG-SHP (2μg plasmid) and wildtype or mutant myc-SHP (3μg plasmid). Plasmid levels were balanced with empty vector. Whole cell extracts were subjected to IP with FLAG antibody. 25% of the immunoprecipitation reaction was resolved by SDS-PAGE and analyzed by western blotting with FLAG and myc antibodies. Signal was quantitated by laser densitometry. Data for mutants are presented as percent of wildtype protein (100%) bound. Values shown are mean ± SD.

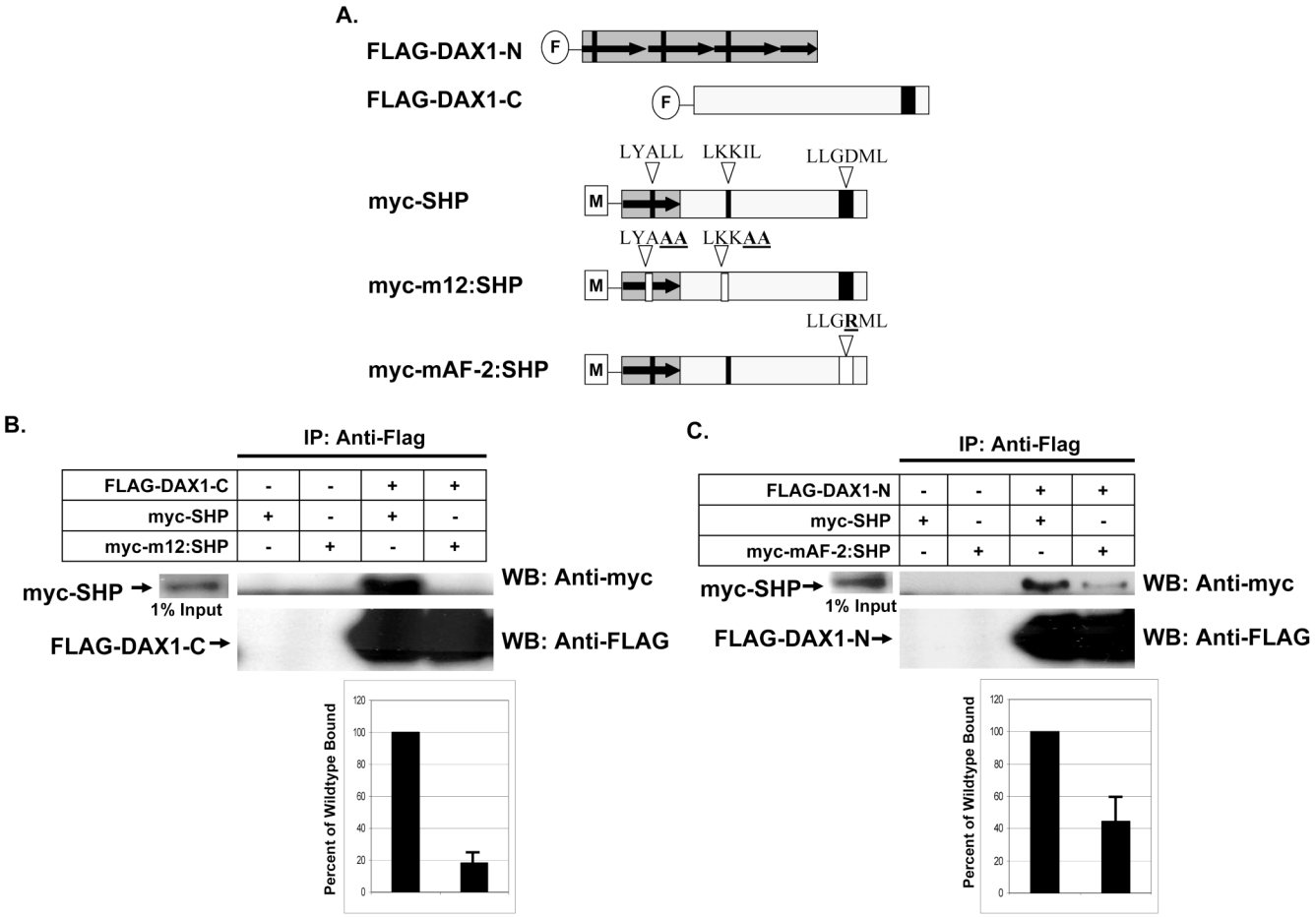
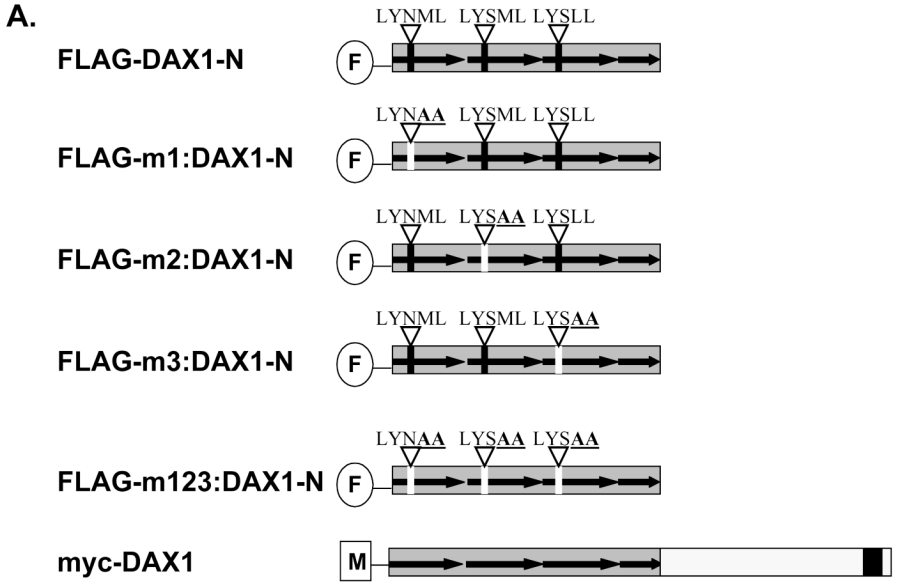


Figure 2. LXXLL motifs and AF-2 domain of SHP are involved in DAX1-SHP heterodimerization

(A) Schematic of FLAG- (F) DAX1 and myc- (M) SHP constructs used in Co-IP assays. LXXLL and AF-2 point mutations are in bold and underlined. Black and white lines represent wildtype and mutant LXXLL motifs, respectively. Black and white boxes represent wildtype and mutant AF-2 domain, respectively. (B and C) SHP LXXLL motif (B) and AF-2 domain (C) involvement in DAX1-SHP heterodimerization. HEK293 cells were transfected with the indicated combinations of FLAG- and myc-epitope tagged expression constructs (2μg each plasmid). Plasmid levels were balanced with empty vector. Whole cell extracts were subjected to IP with FLAG antibody. 25% of the immunoprecipitation reaction was resolved by SDS-PAGE and analyzed by western blotting with FLAG and myc antibodies. Signal was quantitated by laser densitometry. Data for mutants are presented as percent of wildtype protein (100%) bound. Values shown are mean ± SD.



B.

	IP: Anti-Flag				
FLAG-DAX1-N	+	-	-	-	-
FLAG-m1:DAX1-N	-	+	-	-	-
FLAG-m2:DAX1-N	-	-	+	-	-
FLAG-m3:DAX1-N	-	-	-	+	-
FLAG-m123:DAX1-N	-	-	-	-	+
myc-DAX1	+	+	+	+	+

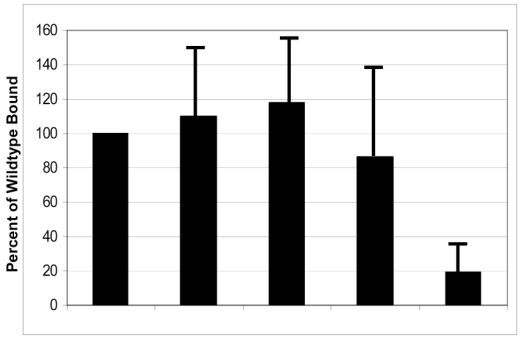


Figure 3. Redundancy of LXXLL motifs in DAX1 homodimerization

(A) Schematic of FLAG- (F) DAX1-N and myc- (M) DAX1 constructs used in Co-IP assays. DAX1-N residue numbers are as in Fig. 3-3. LXXLL point mutations are in bold and underlined, and black and white lines represent wildtype and mutant LXXLL motifs, respectively. (B) Co-IP assay. HEK293 cells were transfected with the indicated combinations of FLAG- and myc-epitope tagged expression constructs (2μg each plasmid). Plasmid levels were balanced with empty vector. Whole cell extracts were subjected to IP with FLAG antibody. 25% of the immunoprecipitation reaction was resolved by SDS-PAGE and analyzed by western blotting with FLAG and myc antibodies. Signal was quantitated by laser

densitometry. Data for mutants are presented as percent of wildtype protein (100%) bound. Values shown are mean ± SD.

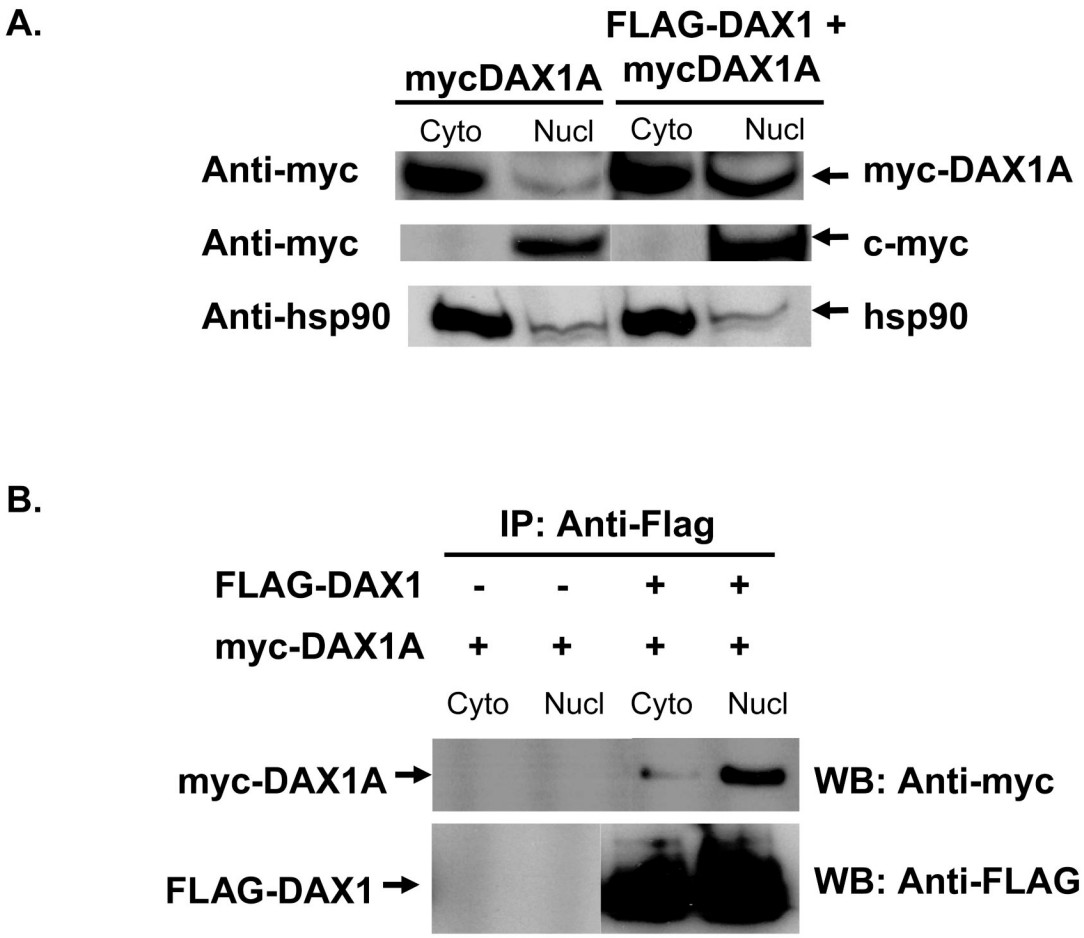


Figure 4. DAX1A subcellular localization is mostly cytoplasmic, but DAX1-DAX1A heterodimers exist in the nucleus

(A) Nuclear and cytoplasmic fractions of HEK293 cells transfected with combinations of FLAG-DAX1 and myc-DAX1A constructs as indicated (2μg each plasmid) were subjected to western blot analysis with FLAG, myc, and hsp90 antibodies. Plasmid levels were balanced with empty vector. Hsp90 and endogenous c-myc protein were used as cytoplasmic and nuclear markers, respectively. DAX1A localization was observed to be primarily cytoplasmic regardless of the presence of DAX1. (B) Nuclear and cytoplasmic fractions of transfected HEK293 cells were immunoprecipitated with FLAG antibody followed by western blot with FLAG and myc antibodies. Co-IP showed that DAX1-DAX1A heterodimerization occurred mostly in the nucleus. Western analysis in (A) represented 1% Co-IP input.

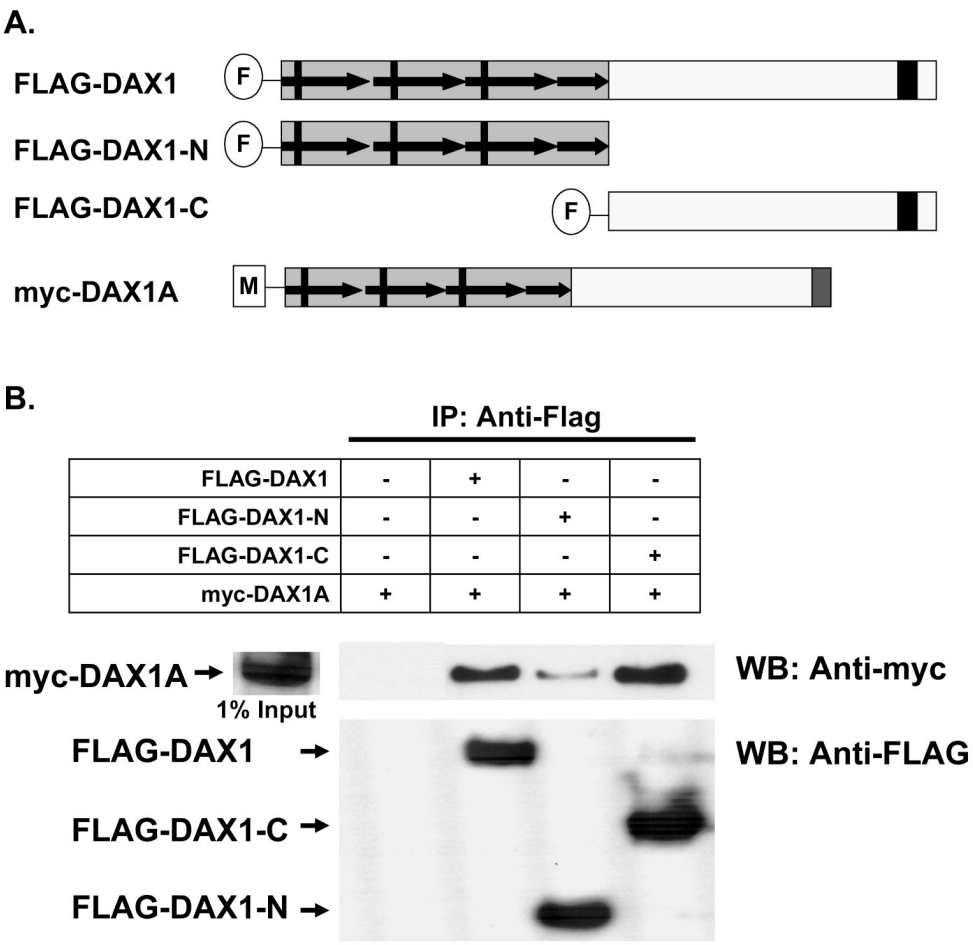


Figure 5. C-terminus of DAX1 is involved in DAX1-DAX1A heterodimerization

(A) Schematic of FLAG-DAX1 (F) and myc-DAX1A (M) expression constructs. DAX1-N contains residues 1-198, and DAX1-C contains residues 207-470. Note the lack of an AF-2 domain in the CTD. DAX1A residues 390-400 comprise a novel 10 amino acid motif (grey bar). (B) Co-IP assays involved transfection of HEK293 cells with indicated combinations of FLAG- and myc-tagged expression constructs (2μg each plasmid). Plasmid levels were balanced with empty vector. Whole cell extracts were subjected to IP with FLAG antibody followed by western blotting with FLAG and myc antibodies.

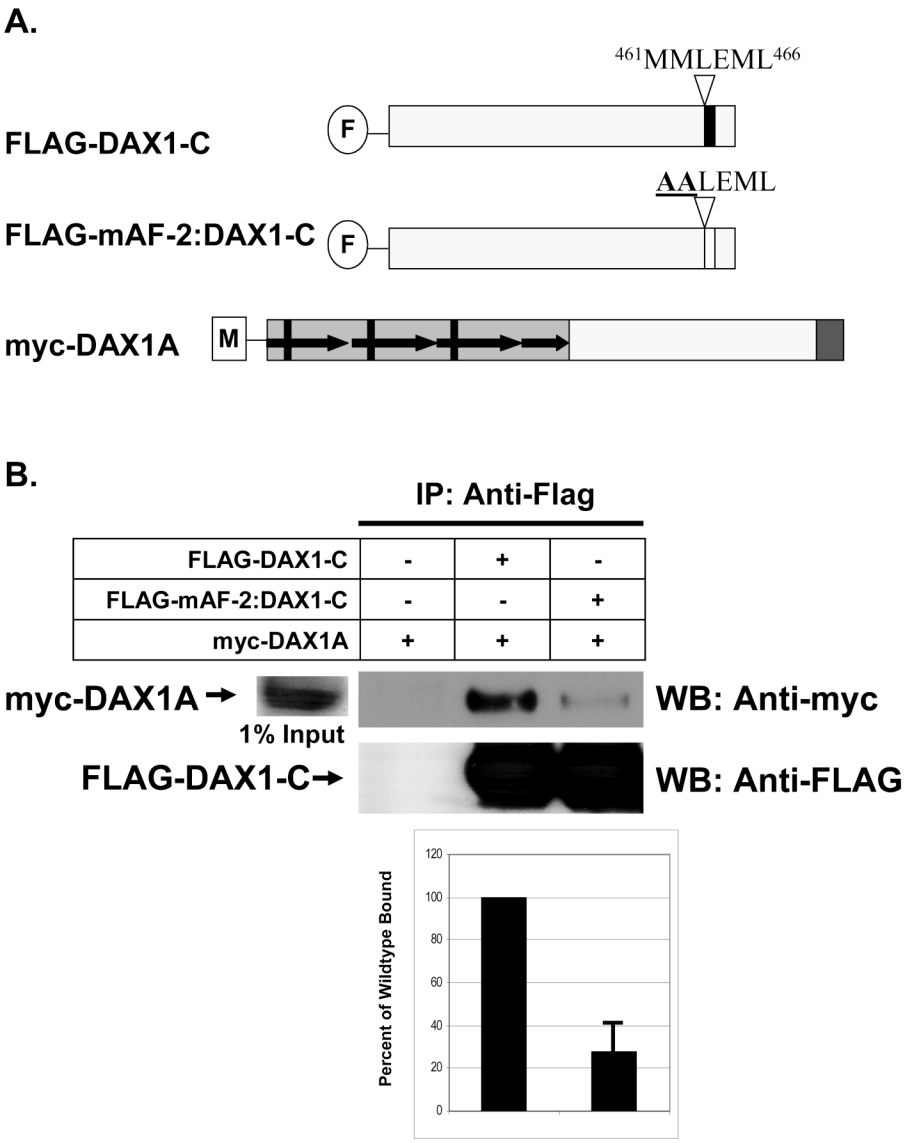


Figure 6. DAX1 AF-2 domain is involved in DAX1-DAX1A heterodimerization

(A) Schematic of FLAG- (F) DAX1 and myc- (M) DAX1A constructs used in Co-IP assays. DAX1-C residue numbers are as in Fig. 5. AF-2 residue numbers are in superscript. AF-2 point mutations are in bold and underlined, and black and white boxes represent wildtype and mutant AF-2 domain, respectively. (B) Co-IP assay. HEK293 cells were transfected with the indicated combinations of FLAG- and myc-epitope tagged expression constructs (2μg each plasmid). Plasmid levels were balanced with empty vector. Whole cell extracts were subjected to IP with FLAG antibody. 25% of the immunoprecipitation reaction was resolved by SDS-PAGE and analyzed by western blotting with FLAG and myc antibodies. Signal was quantitated by laser densitometry. Data for mutants are presented as percent of wildtype protein (100%) bound. Values shown are mean ± SD.