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Ubiquitin tagged dominant negative induces degradation of B-ZIP proteins

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ABSTRACT

B-ZIP transcription factors heterodimerize with dominant negative designs, termed A-ZIPs, in a dimerization specific manner and inhibit its ability to bind DNA. Different A-ZIPs produce unique phenotypes *in vivo* suggesting that they have distinct B-ZIP heterodimerization partners. However, the identification of the *in vivo* heterodimerization partners of different A-ZIPs remains problematic. To identify the *in vivo* heterodimerization partners, a chimeric protein containing two ubiquitin motifs at the N-terminal of the A-ZIP domain was designed. The presence of ubiquitin reduced the concentration of specific co-transfected B-ZIP proteins. The ubiquitin enhanced degradation of the B-ZIP heterodimeric partner is inhibited by the proteasome inhibitor MG-132. These ubiquitin tagged A-ZIP dominant negatives may be more active *in vivo* because their endogenous heterodimerization partners are degraded more efficiently. This may be a general strategy to identify protein interaction partners.

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1. Introduction

The 55 human B-ZIP proteins function as homodimers or heterodimers to bind DNA in a sequence-specific manner and regulate genes involved in cell growth and differentiation [1,2]. To understand the function of structurally related B-ZIP proteins, we have designed dominant negatives, termed A-ZIPs, which contain only the B-ZIP leucine zipper domain and an appended amphipathic α-helical sequence that replaces the B-ZIP basic region [3–7]. The A-ZIP proteins heterodimerize with B-ZIP proteins via their leucine zippers and the acidic extension forms a coiled-coil structure with the B-ZIP basic region, essentially zippers the leucine zipper into the basic region [2]. In our previous studies, we have shown that the inhibition of B-ZIP DNA binding results in physiological effects in transgenic mouse models [8–11].

Expression of the A-ZIP protein A-C/EBP in the epidermis of adult mice prevents skin papilloma formation. If A-C/EBP is expressed after papilloma formation, the papillomas regress [8]. In contrast, A-Fos expression in mammalian cells reduces Ha-*ras*-mediated cellular transformation [4] whereas A-Fos expression in mouse epidermis converts papillomas to benign sebaceous adenomas and prevents conversion into carcinomas [12]. A-CREB expression prevents pappilloma formation but do not cause regression [9]. Taken together, these results suggest that the effective inhibition of B-ZIP DNA binding could be a clinically relevant molecular target.

We observe that the expression of the A-ZIP sometimes causes its *in vivo* B-ZIP target protein expression to be reduced [8] suggesting that the inhibition of B-ZIP DNA binding accelerates degradation. To enhance B-ZIP degradation, we appended two copies of ubiquitin to the A-ZIP. Ubiquitin is a 76-amino acid peptide that is covalently attached to proteins targeting them to the proteasome complex for degradation [13]. By tagging the A-ZIPs with ubiquitin, the B-ZIP|A-ZIP heterodimer may enter the ubiquitin proteasome pathway, leading to the degradation of the heterodimer [14,15]. The induced rapid turnover of pathological B-ZIP proteins may transform ubiquitin–A-ZIPs into more potent dominant negatives.

2. Materials and methods

2.1. B-ZIP, A-ZIP and ubiquitin–A-ZIP Plasmids

The B-ZIP domains of 10 different B-ZIP proteins (ATF2, C/EBPα, C/EBPβ, CREB, cFos, FosB, cJun, JunB, JunD, VBP) containing a green fluorescent protein (GFP) tag were previously designed [16]. Additionally, the A-ZIP domain plasmids were constructed as described previously [17]. Three dominant negative designs (A-FOS, A-C/EBPα, A-CREB) containing plasmids were digested with HindIII and BamHI restriction enzymes. Ubiquitin protein domain was PCR amplified using primers containing HindIII or BamHI restriction enzyme sites and digested with HindIII and BamHI. Gel purified ubiquitin fragments were subsequently ligated into the HindIII-BamHI open site of pT5 plasmid. The resulting plasmid was DNA sequenced to insure both the ubiquitin and A-ZIP design

Abbreviations: B-ZIP domain, basic leucine zipper domain; CREB, CRE-binding protein; VBP, vitellogenin gene-binding protein; C/EBP, CAAT/enhancer binding protein; AP1, activator protein 1.

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was present. After miniprep isolation and small-scale simplification, the ubiquitin–A-ZIP fragment was removed by its HindIII-NdeI sites and inserted into the complementary site on the CMV-500 vector; allowing for efficient cell transfections. The final construct was verified by digestion analysis with the HindIII and NdeI restriction enzymes. The protein sequence of ubiquitin–A-FOS is

*MQIFVKTLTGKTITLEVESSDTIDNVKSKIQDKEGIPPDQQRLIFAGKQLED GRTLSDYNIQKESTLHLVLRLRGG*MQIFVKTLTGKTITLEVESSDTIDNVKSK IQDKEGIPPDQQRLIFAGKQLEDGRTLSDYNIQKESTLHLVLRLTGG**PVAT RDPDLEQRAEELARENEELEKEAEELEQELAELEAETDQLEDEKSALQ-TEIANLLKEKEKL**, (ubiquitin tags in italics and underlined, A-FOS in bold).

2.2. Cell culture and transfections

NIH3T3 (ATCC) cells were maintained in Dulbecco's Modified Eagle Medium with high glucose (Invitrogen), 10% bovine calf serum (Hyclone), and 1% Antibiotic/Antimycotic (Invitrogen) in a 5% CO_2 incubator at 37 °C. The cells were transfected with either GFP-tagged B-ZIP alone or co-transfected with A-ZIP or ubiquitin–A-ZIP. Transfections were carried out following standard protocol (Invitrogen); cells were plated at a density of 6×10^4 on 12-well cell culture clusters (Corning Incorporated) and were transiently transfected with a standardized total DNA plasmid amount (1.3 μg) and lipofectamine (4.0 μl per well) in serum-reduced OPTI-MEM media (Gibco-Invitrogen). After 4 h of transfection, OPTI-MEM media were replaced with serum rich media and cultured for 24 h. Cultures were checked for GFP expression with fluorescence microscopy and harvested 24 h post-transfection using RIPA buffer with a Protease Inhibitor Cocktail Tablet (complete-Mini EDTA-free, Roche). Subsequent BSA protein concentration assays were carried out using standard procedures (Thermo Scientific).

For proteasome inhibition assays, MG-132 (Sigma–Aldrich) was added to the cells at 0, 2.5, 5, and 10 μM concentrations [18]. The compound was suspended in DMSO and added to the cells 4 h post-transfection in fresh media. Controls were kept in DMSO without inhibitor.

2.3. Western blot analysis

Pre-determined amounts of protein were denatured in 1× NuPAGE loading buffer (Invitrogen), 50 mM DTT, and RIPA buffer-Protease inhibitor solution at 70 °C for 10 min. Samples were loaded onto NuPAGE 4–12% Bis–Tris Gel (Invitrogen) and run for SDS electrophoresis (150 V, 90 min). Proteins were then transferred onto a polyvinylidene difluoride membrane (PVDF, Invitrogen) at 30 V for 90 min. The membrane was incubated for 1 h at room temperature in PBS-T (pH7.4, 1× PBS, 0.1% Tween20) containing 5% non-fat dry milk (American Bioanalytical), and then incubated overnight at 4 °C with primary antibodies in milk solution. Antibodies were used for GFP (1:1000 dilution, sc-9996) and the Beta-Actin control (1:2000 dilution, sc-47778). Following the incubations, membranes were rinsed 3 × 10 min with PBS-T. Anti-Mouse IgG secondary antibody (1:5000 dilution, Amersham Biosciences) was suspended in the milk solution and subsequently added to the membrane. The incubation period was kept rocking at RT for 1 h. Membranes were then washed another 3 × 10 min. Chemofluorescence was detected using the ECL Plus western blotting detection kit (Thermo Scientific).

2.4. Reverse transcriptase PCR (RT PCR)

To insure the effects of the dominant negative was not affecting the transcriptional level, the RNA was analyzed for a two-step RT-PCR. RNA was isolated using TRIzol (Invitrogen) using standard protoco. The isolated product was treated with RNase-Free DNase I to digest the DNA impurities and subsequently inactivated by incubating at 65 °C for 5 min. cDNAs were generated using cDNA preparation Kit (Promega) as suggested by the manufacturers protocol. PCR amplifications were performed with the 1 μl of cDNA template of the cJun|FOS system, 4 μl of PCR ReadyMix (Sigma–Aldrich), 3 μl of PCR quality water and 1 μl each of forward and reverse primers. GFP primers, A-FOS primers, and GAPDH primers used are listed below.

GFP Primers:	Forward 5' GCACGACTTCTTCAAGTCCGCCATGCC 3'
	Reverse 5' GCGGATCTTGAAGTTCACCTTGATGCC 3'
A-FOS Primers:	Forward 5' CCGAAGAGCTGGAGCAGGAA 3'
	Reverse 5' TAATCAGGGATCTTGCAGGC 3'
GAPDH Primers:	Forward 5' ATGTTCCAGTATGACTCCACTCACG 3'
	Reverse 5' GAAGACACCAGTAGACTCCACGACA 3'

The reaction conditions were: 1 hold for 9 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 62 °C, and 30 s at 72°C; 1 hold for 7 min at 72 °C. The PCR product was visualized on a 2% agarose gel.

3. Results

3.1. Expression of ubiquitin tagged A-ZIPs

We evaluated if the addition of ubiquitin to the A-ZIP dominant negatives would cause their B-ZIP dimerization partner to be degraded more efficiently. We generated plasmids encoding chimeric proteins termed ubiquitin–A-ZIPs which contained two copies of the 76-a.a. yeast ubiquitin appended to the N-terminus of A-ZIPs (Fig. 1). Three plasmids containing ubiquitin tagged A-ZIP domi-

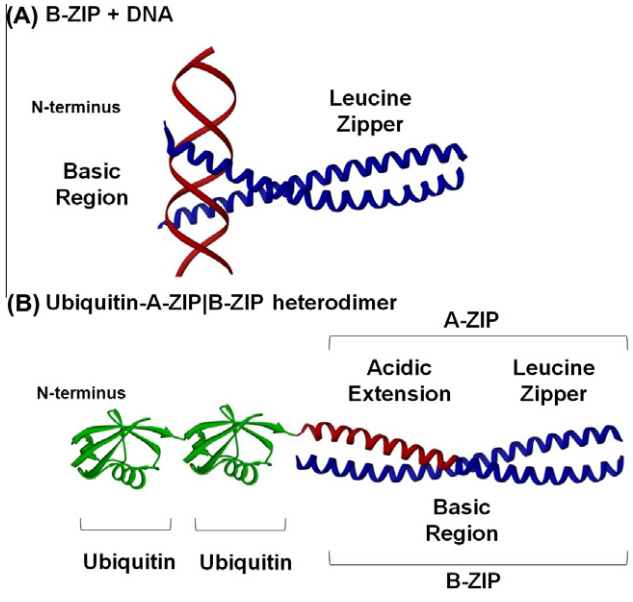

Fig. 1. Schematic representation of A-ZIP dominant negative and ubiquitin–A-ZIP, (A) Structure of B-ZIP domain (Fos|Jun heterodimer (1FOS)) in blue bound to DNA in red. (B) Coiled-coil heterodimer (359 G) between a B-ZIP monomer (blue) and A-ZIP where the acidic extension is in red with two ubiquitin domains (in green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).