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Selected BTB/POZ kelch proteins bind ATP

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Abstract Proteins with a bric-à-brac, tramtrack, broad-complex/Poxvirus zinc fingers (BTB/POZ) domain are implicated in a broad variety of biological processes, including DNA binding, regulation of gene transcription and organization of macromolecular structures. Kelch domain containing BTB/POZ proteins like Mayven and Keap1 display limited sequence similarity with the actin-fragmin kinase from *Physarum*, a protein kinase with a kelch domain. We show that mouse Keap1, a *Caenorhabditis elegans* protein that we named CKR, and human Mayven bind 5′-*p*-fluorosulfonyl-benzoyl-adenosine (FSBA), a covalently modifying ATP analogue. Binding with 2-azido-ATP or ATP-Sepharose is also demonstrated. In contrast to Mayven, FSBA binding by CKR and Keap1 was specifically inhibited by excess ATP. The ATP binding pocket is located in the N-terminal half of Keap1. Our findings indicate that several, but not all, BTB/POZ-kelch domain proteins possess an inconspicuous ATP binding cassette.

Key words: Bric-à-brac, tramtrack, broad-complex; Poxvirus zinc finger; ATP; 5′-*p*-Fluorosulfonyl-benzoyl-adenosine; Modification; Kelch

1. Introduction

BTB/POZ domains (bric-à-brac, tramtrack, broad-complex/Poxvirus zinc fingers) [1,2] are found in actin binding proteins as well as nuclear DNA binding proteins. Members of this family are implicated in the regulation of subcellular localization, DNA binding and gene expression by formation of hetero- or homomeric complexes (reviewed in [3]). The crystal structure of the PLZF (promyelocytic leukemia zinc finger) BTB/POZ domain has been determined [4]. The complex is shown to be a very tight homodimer with an extensive hydrophobic dimerization interface.

Several members of the heterogeneous kelch superfamily contain a BTB/POZ domain in their amino-terminal domain (reviewed in [5]). In the *Drosophila* kelch protein, the BTB/POZ domain is essential for development of the egg chamber ring canals. The amino-terminal BTB/POZ domain enables dimerization of two kelch monomers: the actin binding capacity of the carboxy-terminal kelch repeat can then be used for bundling and stabilizing actin filaments at the inner rim of the canals [6]. The 28 members of the kelch superfamily are characterized by the presence of four to seven repeats of a ~50 amino acid stretch, with eight characteristic signature residues [5]. Based on the crystal structure of the kelch related protein galactose oxidase from *Dactylium dendroides* [7] and sequence similarity [5], the same β-propeller superfold is predicted for all other kelch repeat domains. Viral neuraminidases [8–10], heterotrimeric G-protein β-subunits [11] and WD repeat containing proteins in general [12] all show a similar β-propeller superfold.

Actin-fragmin kinase (AFK) of the slime mold *Physarum polycephalum* also contains a kelch domain [13]. This protein kinase phosphorylates actin on Thr203 [14] when one of the fragmin isoforms (P, A or 60) binds actin in a 1:1 complex [14–17]. Comparison between the catalytic subunits of PKA [18] and AFK [19] showed conservation of barely five residues in the core of AFK. These residues are involved in phosphoryl transfer. Apart from this strict conservation, the AFK catalytic domain shows no similarity with other protein kinases. Detailed analysis of the similarities between AFK and kelch related proteins pointed out a weak but extended sequence similarity with the amino-terminal half of the BTB/POZ domain containing proteins Mayven (human), Keap1 (mouse) and *Caenorhabditis elegans* yk103a10. Based on this limited homology, we set out to investigate if the *C. elegans* protein (CKR), Mayven or Keap1 share biological properties with the AFK.

2. Materials and methods

Glutathione-Sepharose 4B, secondary HRP conjugated antibodies, and Hybond C nitrocellulose membrane were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). NBT/BCIP and GAR alkaline phosphatase conjugated IgG were from Duchefa (Haarlem, the Netherlands) and from Sigma (St. Louis, MO, USA), respectively. Easy start tubes and the QuickChange mutagenesis kit were from Molecular Bio-Products (San Diego, CA, USA) and Stratagene (La Jolla, CA, USA), respectively. Platinum Taq and lipofectamine were from Gibco BRL Life Technologies (Rockville, MD, USA). Complete® inhibitor cocktail tablets were from Boehringer Mannheim (Mannheim, Germany). FSBA, NTPs, GTP-Sepharose and ATP-Sepharose were purchased from Sigma. 2-Azido-[^{32}P]ATP was from ICN (Costa Mesa, CA, USA).

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Abbreviations: AFK, actin-fragmin kinase; BTB/POZ, bric-à-brac, tramtrack, broad-complex/Poxvirus zinc fingers; CKR, *Caenorhabditis elegans* kelch related; FSBA, 5′-*p*-fluorosulfonyl-benzoyl-adenosine; PMSF, phenylmethylsulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

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2.1. cDNA cloning

2.1.1. CKR. The 5′ side of the T27e9.4 cosmid contains three ATG codons. Separate PCR reactions were performed on 1 μg of a yeast two-hybrid cDNA library from *C. elegans* adults (a kind gift of Dr. Ir. Wim Van Crieckinge, DevGen, Gent, Belgium) according to the following schedule: 3 min denaturation, 1 min denaturation at 94°C, 1 min annealing at 60°C, and extension for 30 s at 72°C (2.5 units Platinum Taq DNA polymerase, 0.4 μM primers, 30 cycles). Only the primer combination 5′-GGATCCCGGCATTCTCAATGTTCGCATCTG-3′ (corresponding with the third ATG codon) and 5′-GCGGCCGCTCACACATATGAGGTGGC-ATCGG-3′ resulted in a ~1.8 kb cDNA.

The cDNA clone yk103a10 encoding CKR was also obtained by in vivo excision from a Lambda ZAP II phagemid (obtained from Dr. Yuji Kohara, National institute of Genetics, Japan) according to instructions of the manufacturer. The cDNA obtained from different sources were both sequenced and showed complete sequence identity.

2.1.2. Keap1. Keap1 cDNA was amplified by PCR using a mouse brain cDNA library as template (a kind gift of Dr. Bart Destrooper, KUL) using the primers 5′-GGAATTCATGCAGCCCGAACCCAAGCTTAGCGG-3′ and 5′-GCTCGAGTTTATTAGCAGGTACAGTTTTGTTGATC-3′. 25 cycles were performed with 2.5 units of Platinum Taq (94°C, 1 min; T_{an}: 62°C, 1 min; T_{ext}: 72°C, 1 min) yielding a product of about 1.8 kb. The PCR fragment was cloned as an *Eco*RI/*Xho*I fragment into pGEX-5X-1 (Amersham Pharmacia Biotech).

The amino-terminal half of Keap1 (amino acids 1–340) was cloned separately into pGEX-5X-1.

2.1.3. Mayven. The cDNA coding for Mayven was amplified by PCR from a human fibrosarcoma cDNA library (2fTGH cells; library constructed by Els Pattyn, Department of Biochemistry, Faculty of Medicine and Health Sciences, Ghent University, Belgium). The primers used were 5′-GGAATTCATGGAGACGCCGCCGCTGCCTCCCG-3′ and 5′-AGCGGCCGCCTAATGGTTTATCAATAACTGTGACC-CCTG-3′. The 1.5 kb cDNA was cloned into pGEX-5X-1.

2.2. Purification of recombinant GST fusion proteins

An overnight culture (250 ml Luria-Bertani broth (LB), 100 μg/ml ampicilin) of *Escherichia coli* MC1061 cells containing the pGEX-5X-1 plasmids coding for CKR, Keap1 or Mayven was used to inoculate a culture of 5 l. Cells were grown until $OD_{595nm} = 0.8$ was reached; expression was induced with 0.5 mM IPTG (Eurogentec, Seraing, Belgium) and the cells were incubated for 20 h at 22°C. After centrifugation at 3000×*g* for 5 min, pellets were resuspended in ice-cold lysis buffer (20 mM Tris pH 7.5, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF) and Complete™ protease inhibitor cocktail tablets). Lysis was performed by passing the cells twice through a French press (AMINCO, Illinois, USA). For optimal extraction, Triton X-100 was added to the supernatant to a final concentration of 1%. The lysate was centrifuged for 1 h at 100 000×*g* (4°C). Purification on glutathione-Sepharose was performed according to instructions of the manufacturer, and appropriate fractions were concentrated using a SpeedVac concentrator (Savant Instruments, Holbrook, NY, USA). To remove impurities, proteins were further purified to near homogeneity on a Superdex 200 gel filtration column (Amersham Pharmacia Biotech) equilibrated in 10 mM HEPES pH 7.4, 25 mM NaCl and 0.02% sodium azide at a flow rate of 0.3 ml/min.

2.3. FSBA binding assays

1 μg protein was incubated with 0.5 mM 5′-*p*-fluorosulfonyl-benzoyl-adenosine (FSBA) in a total volume of 25 μl in the presence of 5 mM $MgCl_2$ and 100 mM HEPES, pH 7.2 (to avoid a drop in pH when ATP was added at elevated concentrations). In competition experiments ATP or other nucleotides were included. Reactions were terminated by the addition of 5× Laemmli sample buffer [20]. Samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto nitrocellulose membranes. BSA depleted anti-FSBA antibodies (generously donated by Dr. M. Wymann, Institute of Biochemistry, University of Fribourg, Fribourg, Switzerland) were added at a 1:2000 dilution, and binding

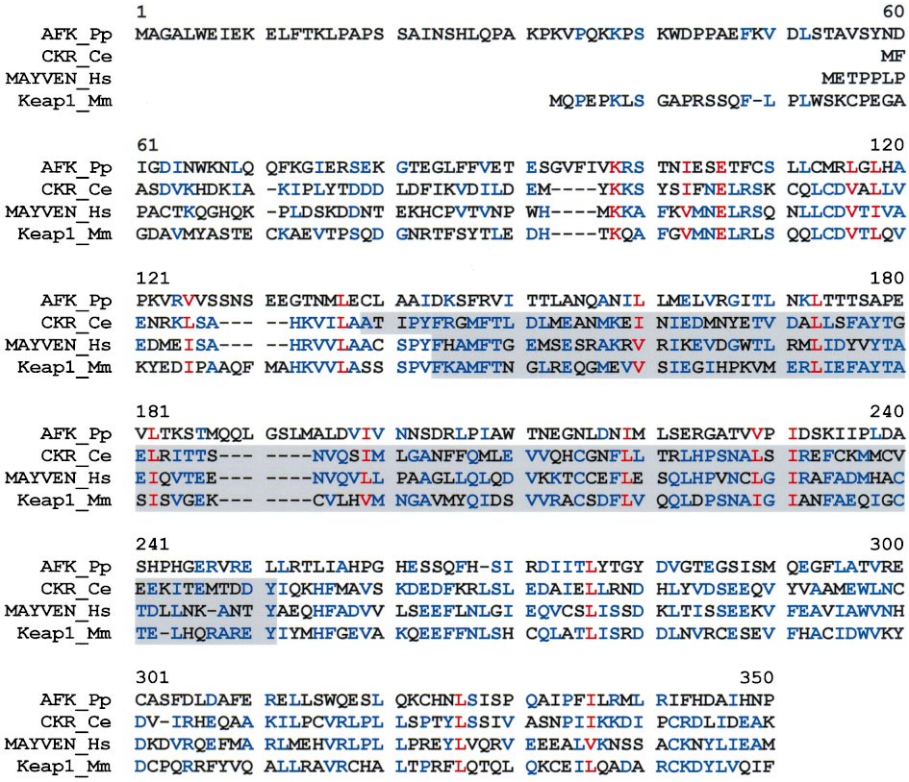

Fig. 1. Alignment between AFK (N-terminal half) and BTB/POZ-kelch proteins (N-terminal half). Pp, *P. polycephalum*; Ce, *C. elegans*; Hs, *Homo sapiens*; Mm, *Mus musculus*. Amino acids that are conserved in all four proteins are shown in red, amino acids that are conserved in two or three members are depicted in blue. The shaded region is the BTB/POZ domain.

was visualized following incubation with secondary anti-rabbit HRP coupled antibodies and ECL detection reagents.

2.4. ATP-Sepharose binding assays

3 μg CKR was incubated with 10 μl settled GTP-Sepharose or ATP-Sepharose in 10 mM HEPES pH 7, 50 mM KCl, 10 mM $MgCl_2$, 1 mM DTT and 0.2% Triton X-100 overnight at 4°C in a total volume of 300 μl. Following three washes with the same buffer, the pellet was boiled in Laemmli sample buffer and analyzed by SDS–PAGE, followed by Coomassie staining or Western blotting with anti-GST antibodies.

2.5. Immunoprecipitation of GFP-Keap1

The Keap1 cDNA was cloned into pEGFP-N1 (Clontech) as an *Eco*RI/*Bam*HI fragment. MDCK-AZ cells [21] were transfected with GFP tagged Keap1 or GFP (control) using lipofectamine. For immunoprecipitation, cells were washed twice with ice-cold TBS (20 mM Tris, 150 mM NaCl, pH 7.4) and lysed in 20 mM Tris pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM NaF, 1 mM sodium pyrophosphate, 5 mM levamisole, 8 mM β-glycerophosphate and Complete™ protease inhibitor cocktail tablets, 1% Triton X-100 and 0.5% NP40. Cells were disrupted by sonication in a Vibra Cell W tip sonicator (Sonics and Materials Inc., Danbury, CT, USA) set at 40% duty cycle. The extract was centrifuged 20 000×*g* at 4°C for 15 min. Following overnight incubation of antibodies and cell lysate at 4°C, beads were recovered by centrifugation and the pellet was washed three times with ice-cold TBS supplemented with 1% Triton X-100 and 0.5% NP40 and once with TBS without detergents. SDS sample buffer was added and samples were boiled for 5 min followed by SDS–PAGE analysis and Coomassie staining.

2.6. Miscellaneous

Protein concentrations were determined by the method of Bradford [22] using bovine serum albumin as standard. SDS–PAGE was carried out according to [23]. Western blot analysis was performed as described by Towbin et al. [24]. Total lab software for quantitation of autoradiograms was from Phoretix (Newcastle upon Tyne, UK). Multiple sequence alignments were performed using the Multalin Program (http://prodes.toulouse.inra.fr/multalin/multalin.html) [25].

3. Results and discussion

3.1. Database screening for AFK homologs

Available protein and DNA databases were screened with the blastp algorithm [26] to search for homologs of the *P. polycephalum* AFK. The *C. elegans* wormpep database (http://www.sanger.ac.uk) yielded a predicted protein that revealed similarity to both the carboxy-terminal (23% identity, 38% positives in a 130 amino acid stretch) and amino-terminal domains of AFK (22% identity, 47% positives on a 44 amino acid stretch). AFK residues K98 and E106, known to be involved in ATP binding [19], were conserved in the *C. elegans* protein (Fig. 1). Similar entries in human, mouse and rat sequence databases were found and these proteins include a BTB/POZ domain in the N-terminal half and a C-terminal kelch domain: they include the human Mayven protein [27], mouse and rat Keap1 [28], and the predicted human genes KIAA1490 and KIAA1687.

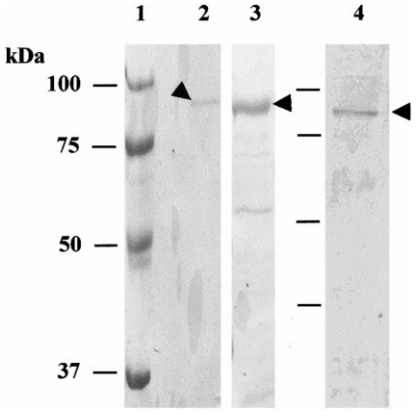

Fig. 2. Purification of kelch domain containing BTB/POZ proteins. SDS–PAGE (10%) analysis of purified GST fusion proteins (arrowheads). *C. elegans* kelch related protein (lane 2), Keap1 (lane 3) and Mayven (lane 4) were purified by additional gel filtration chromatography. Molecular mass markers are shown on the left. The gel was stained with Coomassie brilliant blue.

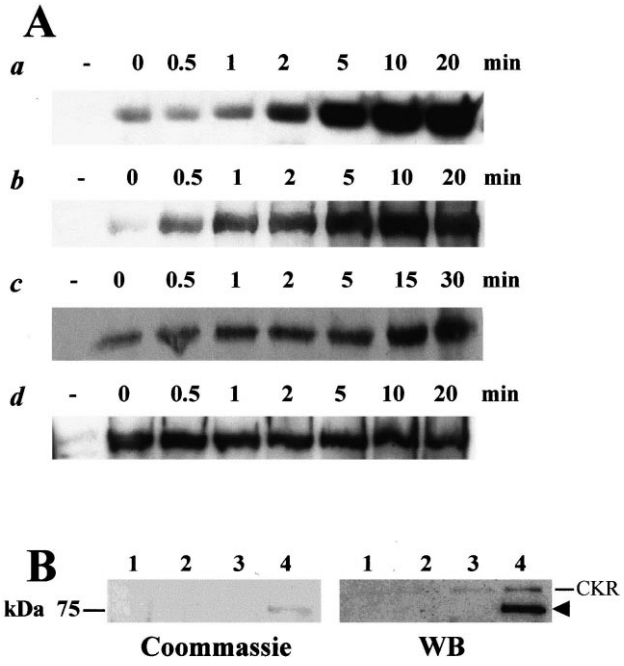

Fig. 3. BTB/POZ-kelch domain proteins bind FSBA and are retained by ATP-Sepharose. A: Proteins (0.5 μM) were incubated with FSBA (0.5 mM) for the indicated periods of time, subjected to SDS–PAGE and Western blotted with anti-FSBA antibodies. a, AFK; b, CKR; c, Keap1; d, Mayven. −, no addition of FSBA. Note that Mayven shows no time dependent increase in FSBA binding. B: CKR was incubated with Sepharose (lane 1), GTP-Sepharose (lane 2), ATP-Sepharose in the presence of 1 mM free ATP (lane 3) or with ATP-Sepharose (lane 4). Left panel, Coomassie staining; right panel, Western blot with GST antibodies. The full length protein is indicated as well as the CKR breakdown product (arrowhead).

3.2. Purification of CKR, Mayven and Keap1

The three kelch domain containing proteins were expressed in *E. coli* as fusion proteins with glutathione-*S*-transferase and purified by affinity chromatography (Fig. 2). Expression in insect cells produced extremely low yields. Subsequent Superdex 200 gel filtration analysis was performed to remove impurities, revealing that all three proteins form oligomers (not shown) because they eluted at a molecular mass far above their expected molecular mass as a monomer (Keap1: 68 kDa, Mayven: 65 kDa and CKR: 65 kDa). Homo-multimerizing properties and similar elution profiles on gel filtration chromatography have been described previously for the BTB/POZ domain containing proteins calicin and the GAGA transcription factor [29,30].

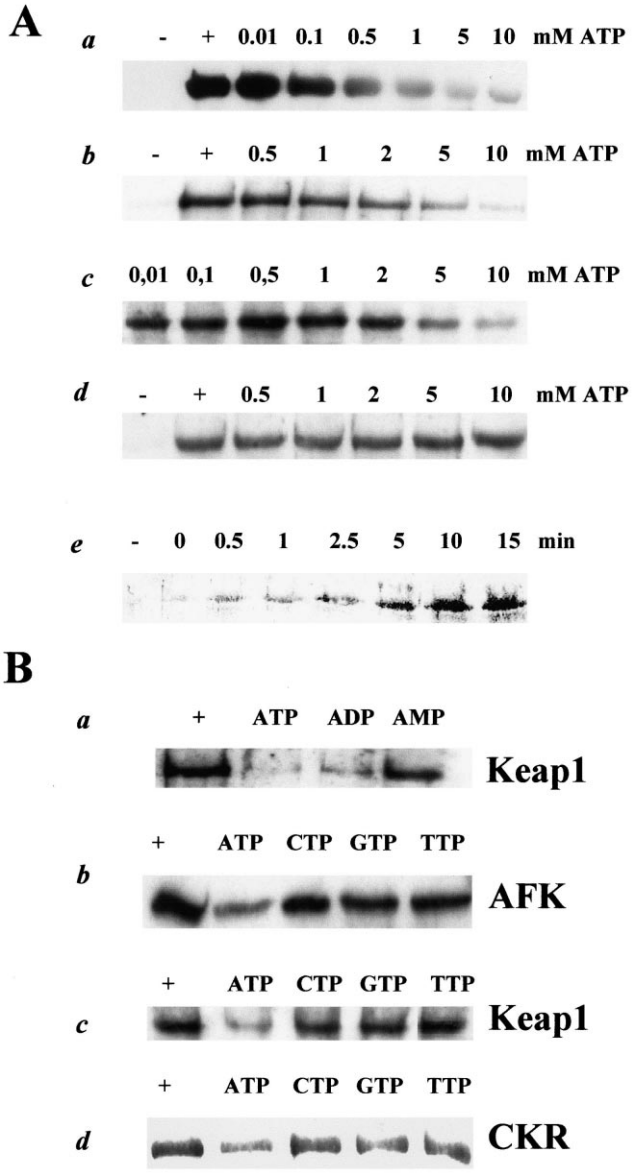

Fig. 4. Competition experiments between ATP and FSBA, and nucleotide specificity. A: 0.5 μM protein was incubated with 0.5 mM FSBA with or without increasing ATP concentrations. (a) AFK = positive control. Increasing ATP concentrations inhibit FSBA binding by CKR (b) and Keap1 (c). For (d) Mayven, excess ATP has no inhibitory effect. (e) Time course of FSBA binding by the Keap1 K61R mutant. −, no addition of FSBA; +, addition of FSBA only. B: Nucleotide specificity of BTB/POZ proteins. Competition between FSBA and (a) ATP, ADP or AMP for binding to Keap1 or (b) ATP, CTP, GTP or TTP for binding to AFK, CKR or Keap1. Nucleotides were used at a 20-fold molar excess in (a) and 10-fold molar excess in (b). The inhibitory effect is more pronounced for ATP. +, FSBA without addition of nucleotide.

3.3. Binding of FSBA by CKR, Mayven and Keap1 is competed specifically by ATP

A minimal requirement for conventional protein kinases and other nucleotide binding proteins is that they bind ATP. Because the amount of recombinant protein obtained was insufficient for studies using fluorescent MANT-ATP binding, we employed FSBA, a covalently modifying ATP analog, that has been used extensively to study ATP binding by a variety of proteins such as lipid kinases [31], 5′-lipoxygenase [32], isocitrate dehydrogenase kinase/phosphatase [33] and the nuclear protein CHL1 [34]. Binding of FSBA was monitored by Western blotting using anti-FSBA antibodies previously employed to study PI3K [31].

Control experiments with recombinant AFK showed that incubation with a constant amount of FSBA resulted in a time dependent binding of the nucleotide analog (Fig. 3A, a). Similar experiments with the BTB/BOZ-kelch domain containing proteins showed that CKR and Keap1 also bound FSBA in a time dependent manner (Fig. 3A, b and c). On the other hand, Mayven showed binding kinetics different from CKR and Keap1 (Fig. 3A, d) and this pattern was similar to non-specific binding of FSBA by serum albumin (not shown).

Incubation of CKR with ATP-Sepharose or GTP-Sepharose showed specific retention of the protein onto ATP-Sepharose (Fig. 3B, left panel, lane 4), although some degradation had occurred during incubation. No binding was observed when free ATP was included in the mixture (Fig. 3B, left panel, lane 3). Western blotting with GST antibodies confirmed the identity of CKR (Fig. 3B, right panel). A similar pattern was obtained with Keap1 (not shown).

To investigate the binding specificity we performed competition experiments between FSBA and ATP. Since FSBA is a covalently modifying ATP analog, incubation periods were kept short (5 min) to avoid saturation by FSBA. When mixtures of FSBA and ATP were added to the AFK we observed ATP mediated concentration dependent inhibition of FSBA binding (Fig. 4A, a). This is in agreement with the presence of an ATP binding site in the N-terminal catalytic domain of AFK [19]. An equimolar ratio of FSBA and ATP resulted in ~50% inhibition of FSBA binding whereas a 20-fold molar excess of ATP caused ~90% inhibition. FSBA binding by CKR and Keap1 was equally inhibited by increasing ATP concentrations: 10 mM ATP caused ~90% inhibition for CKR and ~75% inhibition for Keap1 (Fig. 4A, b and c).

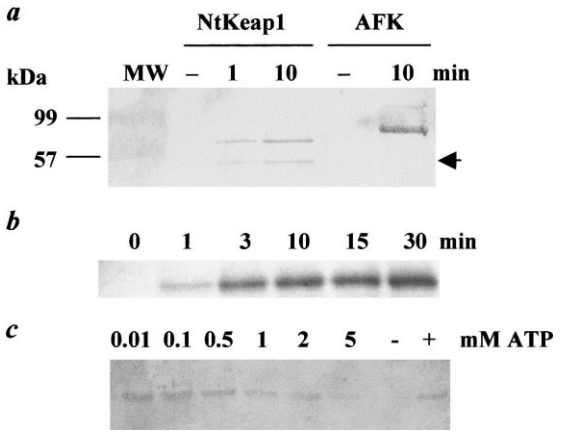

Fig. 5. The Keap1 N-terminal half binds ATP. Time dependent incubation (a and b) and ATP/FSBA competition experiment (c) with recombinant amino-terminal half of Keap1 (NtKeap1) demonstrate that ATP binding properties of the N-terminal half are similar to those of the entire protein. (a) shows FSBA binding of NtKeap1, revealed with anti-FSBA antiserum; (b) shows time dependent association of NtKeap1 with 2-azido-[^{32}P]ATP. (c) was developed as (a). AFK was included as a positive control. −, no FSBA. The arrowhead shows NtKeap1. The protein above NtKeap1 was identified as *E. coli* DNA K and co-purified with NtKeap1.

However, FSBA binding to Mayven was not inhibited, even at the highest ATP concentrations (Fig. 4A, d). Since binding of FSBA by serum albumin is not inhibited by ATP either (not shown), we deduce that Mayven associates with FSBA and ATP in a non-specific manner. On the other hand, the inhibitory effect of ATP on covalent modification of CKR and Keap1 indicates that they bind ATP specifically.

Amino acids that are conserved in the amino-terminal half of AFK, Keap1 and CKR include residues Lys98 and Glu106. K98 is involved in orienting the α-phosphate group of ATP, and forms an additional salt bridge with E106. To investigate the effect of these amino acids on ATP binding, the corresponding amino acids in Keap1 (K61R and E69A) and CKR (K35R, K36R and E43G) were substituted. However, all mutants bound FSBA with similar kinetics (shown for the K61R mutant of Keap1, in Fig. 4A, e) as the wild type proteins. We subsequently investigated whether the mutations affect putative enzymatic properties of the kelch related proteins. However, (auto)phosphorylation or ATPase activity could not be demonstrated. ATP binding had also no effect on oligomerization (not shown).

3.3.1. Inhibitory effect of other nucleotides on FSBA binding by BTB/POZ proteins. Competition assays further showed that adenosine monophosphate (AMP) and adenosine diphosphate (ADP) were less effective than ATP in blocking FSBA modification of Keap1 (Fig. 4B, a). To further test the nucleotide binding specificity of BTB/POZ proteins, comparative experiments were performed with ATP, GTP, CTP and TTP. All were used at 10-fold molar excess to FSBA. In each case, ATP showed stronger inhibitory activity towards FSBA binding than the other nucleotides (Fig. 4B, b–d). From these experiments we conclude that CKR and Keap1 display the highest affinity for ATP.

3.3.2. The N-terminal half of Keap1 binds ATP. To establish whether the amino-terminal half of Keap1 (amino acids 1–340, containing the BTB/POZ domain) is necessary and sufficient for ATP binding, we expressed and purified this construct separately. Incubation with FSBA showed a time

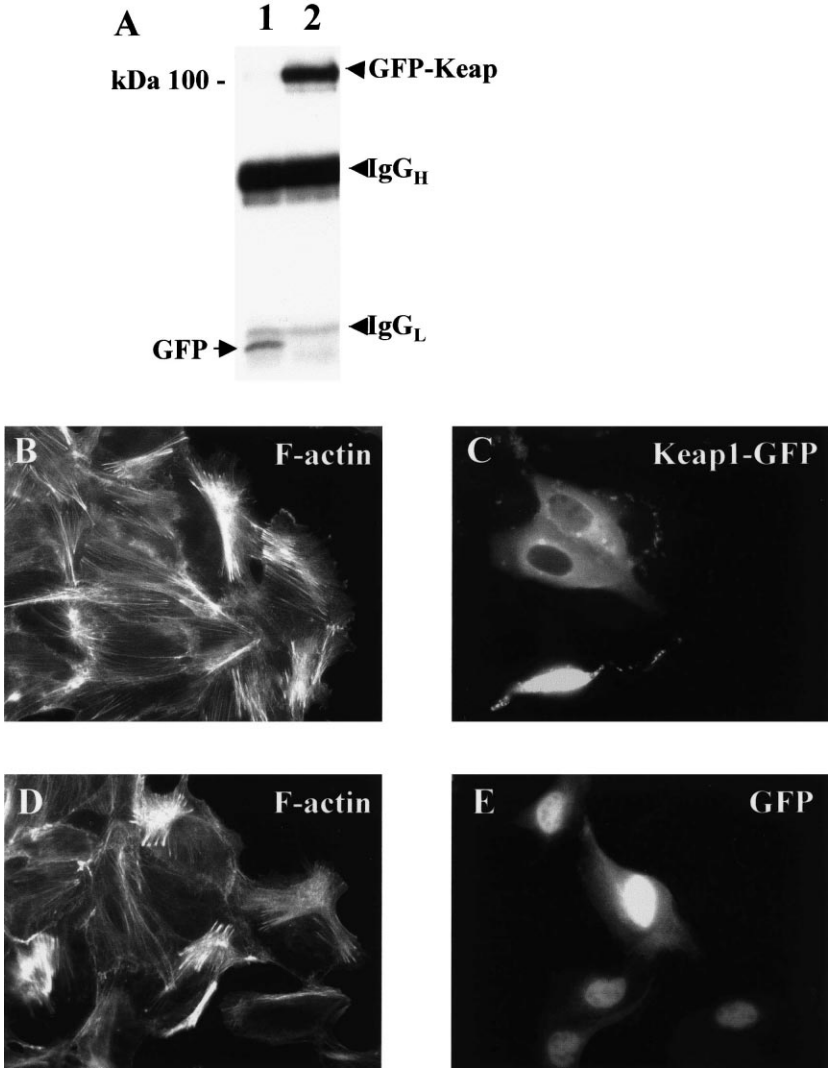

Fig. 6. Keap1 does not co-localize with filamentous actin. A: Detection of GFP-Keap1. Lane 1, control: detection of GFP following immunoprecipitation from transiently transfected MDCK-AZ cells and Western blotting with anti-GFP antibodies. GFP is indicated by the arrow. Lane 2, the same experiment as in lane 1 but with MDCK-AZ cells transfected with GFP tagged Keap1. B–E. Immunofluorescence images of MDCK-AZ cells transiently transfected with GFP-Keap1 (C) or GFP control (E) and stained for F-actin with TRITC-phalloidin (B, D). GFP-Keap1 is diffusely distributed throughout the cytosol; GFP stains the nucleus and cytosol.

dependent increase in binding (Fig. 5a) as revealed with the FSBA antiserum. In addition, crosslinking of NtKeap1 with 5 μM 2-azido-[^{32}P]ATP also showed an increase in binding over time (Fig. 5b). ATP mediated inhibition of FSBA binding (Fig. 5c) was similar to what was found for the full length protein. This result illustrates that intrinsic ATP binding activity resides in the N-terminal half of the protein.

3.4. Keap1 is a cytosolic protein that does not interact with actin

Actin binding has been demonstrated for the kelch domains of the *Drosophila* kelch protein [6], scruin [35–38], ENC-1 [39], Mayven [27] and calicin [29]. Based on these findings, there is a tendency to assume that all kelch domains bind actin. We tested this hypothesis for Keap1.

Identification of Keap1 binding partners was pursued by co-immunoprecipitation of GFP-Keap1 from transfected MDCK cells. Immunoprecipitation of GFP-Keap1 using anti-GFP antibodies followed by Western blotting with anti-actin antibodies did not show co-immunoprecipitation with actin (data not shown). Probing the blot with anti-GFP antibodies revealed a protein of approximately 100 kDa showing that the fusion protein was expressed (Fig. 6A, lane 2). GFP-Keap1 transfected MDCK cells showed a diffuse cytoplasmic staining of the fusion protein (Fig. 6C) with no apparent co-localization with actin stress fibers or cortical F-actin and this was confirmed by superposition of the staining patterns (not shown). We therefore conclude that the Keap1 kelch domain, in contrast to several other kelch domains such as Mayven [27] or *Drosophila* kelch [6], does not bind actin under the conditions studied here. We do not exclude however that, under altered conditions, Keap1 could associate with actin.

This is the first report showing that kelch proteins with a BTB/POZ domain contain a cryptic nucleotide binding cassette specific for ATP. Our results suggest that only selected BTB/POZ-kelch proteins are characterized by this property since Mayven was shown to bind FSBA in a non-specific manner. At present, it is not clear which residues in the N-terminal half of Keap1 or CKR might be responsible for selective nucleotide binding. A very conserved ATP binding motif is the Rossman fold, but analysis of the PLZF BTB/POZ domain crystal structure [4] does not show a typical beta-alpha-beta-alpha-beta structure, characteristic of ATP binding proteins. ATP binding is not associated with intrinsic (auto)phosphorylation or ATPase activity of the BTB/POZ-kelch proteins that were tested. We cannot exclude at this stage that a co-factor of BTB/POZ-kelch proteins could trigger ATPase activity. Alternatively, ATP binding might affect the stability of the protein or its interaction with ligands.

In conclusion, the observation that BTB/POZ proteins contain a nucleotide binding pocket adds a new element to this field of research and opens interesting new prospects for studying the biology of this extended class of proteins and their pleiotropic effects on the regulation of gene expression.

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