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A20 Inhibits NF-κB Activation Independently of Binding to 14-3-3 Proteins

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The A20 protein, which belongs to a class of Cys_2/Cys_2 zinc finger proteins, has been characterized as an inhibitor of NF-κB activation. In order to clarify its molecular mechanism of action, the yeast two-hybrid system was used to screen for interacting proteins. We report that different isoforms of 14-3-3 proteins, viz. η and ζ, are able to bind A20, involving the 14-3-3-binding motif RSKSDP located between zinc fingers 3 and 4. However, A20 mutants that no longer associated with 14-3-3 proteins could still fully inhibit NF-κB activation induced by tumor necrosis factor, interleukin-1β or phorbol 12-myristate 13-acetate, thus excluding a crucial role for 14-3-3 interaction in this A20 function.

A20 is a primary response gene which was originally identified as a cytokine-inducible gene in human umbilical vein endothelial cells (1). Subsequent research demonstrated that A20 is not only induced in endothelial cells by bacterial lipopolysaccharide or the cytokines tumor necrosis factor (TNF) and interleukin-1 (IL-1), but also by other stimuli in other cell types. These include the Epstein-Barr virus LMP1 gene product (2) and the B-cell surface receptor CD40 (3) in B-cells, as well as the human T-cell leukemia virus type I Tax and phorbol 12-myristate 13-acetate (TPA) (4) in Jurkat cells. Superinduction of the A20 gene can often be observed by costimulation with cycloheximide. Remarkably, the same gene has been cloned as a monocyte adherence cDNA clone, and is therefore also called MAD6 (5). Besides its initial characterization as an inhibitor of cell death (6), A20 is a potent cellular inhibitor of NF-κB activation (7-9).

The molecular mechanism of action responsible for A20-mediated inhibition of NF-κB activation is at present largely unknown. The human A20 gene encodes a 790 amino acid-containing protein, consisting of a C-terminal domain with seven Cys_2/Cys_2 zinc fingers (10). In accordance with several known zinc finger proteins (11), A20 has been postulated to function as a transcriptional modulator (10), but specific DNA binding has never been demonstrated. Recently, using the yeast two-hybrid system, we and others demonstrated that A20 is able to self-associate by its zinc finger domain (8, 12), pointing at a role for the seven Cys_2/Cys_2 zinc fingers in protein-protein interaction. Indeed, a heterotypic protein-protein interaction between A20 and the TNF receptor-associated proteins TRAF1 and TRAF2 has been described (8). In addition, while this work was in progress, an interaction between A20 and several isoforms of 14-3-3 proteins was also reported (13). A20 thus joins the growing list of 14-3-3-binding proteins mainly involved in multiple signaling pathways (14). Recently discovered members of 14-3-3-binding proteins include the cell death agonist Bad (15), Wee1 kinase (16) and the glucocorticoid receptor (17). Considering that 14-3-3 proteins dimerize (18) and in view of their crystal structure (19, 20), it is likely that these 28-30 kDa proteins function as adaptor and scaffold proteins within large protein complexes. Detailed analysis of the interaction between 14-3-3 proteins and the serine/threonine kinase Raf-1 revealed that 14-3-3 interaction is mediated by phosphorylation of a serine residue within a conserved RSXpSXP motif (21), although alternative 14-3-3 interaction sequences have been proposed (22, 23). In this report we pin-pointed the 14-3-3-binding site within A20 to the RSKSDP sequence located between zinc fingers 3 and 4. By site-directed mutagenesis of this motif we demonstrate that disruption of the interaction between A20 and 14-3-3 proteins does not result in loss of A20-mediated inhibition of NF-κB activation.

MATERIALS AND METHODS

Cell line and reagents. Human embryonic kidney HEK293T cells were a kind gift of Dr. M. Hall (Department of Biochemistry,

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University of Birmingham, UK). Optimem and lipofectamine were obtained from Life Technologies (Paisley, UK). TPA was purchased from Sigma Chemical Co. (St Louis, MO). Monoclonal anti-E-tag antibody was obtained from Pharmacia Biotech (Uppsala, Sweden) and polyclonal anti-14-3-3β antibody from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and anti-rabbit horse-radish peroxidase-linked antibodies, as well as an ECL Western blotting detection kit were purchased from Amersham Life Science (Amersham, UK). Protein A-linked trisacryl beads were from Pierce Chemicals (Rockford, IL).

Yeast two-hybrid system. A Matchmaker yeast two-hybrid system was purchased from Clontech Laboratories (Palo Alto, CA). L929r2 cDNA library screening or cotransformation of putative interacting proteins was performed as described earlier (12). cDNA inserts encoding candidate A20-associating protein fragments were sequenced on both strands with a cycle sequencer (Applied Biosystems, Foster City, CA). BLAST searches were conducted using the NCBI on-line service.

Generation of A20 muteins. The C-terminal-deleted A20 cDNA (A20ZF$^-$) spans amino acids 1-367 and lacks the seven Cys_2/Cys_2 zinc fingers. The N-terminal-deleted A20 cDNA (A20ZF$^+$) spans amino acids 373-790 and contains the seven Cys_2/Cys_2 zinc fingers. Their construction has been described previously (12).

Site-directed mutagenesis of A20 cDNA was carried out with a kit from Clontech Laboratories in pMa containing a chloramphenicol-sensitive gene (24). By using either the R562A primer CCTTCCTGTCACCAGGCTAGCAAGTCAGATCCC or the S565A primer GTCACCAGCGATCGAAGGCAGATCCCTCG, as well as a second primer which activates the chloramphenicol-resistance gene, one can select for mutants by growing transformants in the presence of chloramphenicol. Generated mutations were verified by DNA sequencing and were cloned in-frame of the GAL4 DNA-binding domain into the yeast expression vector pAS2, or after the E-tag into the eukaryotic expression vector pCAGGS (25).

Coimmunoprecipitation and Western blotting. HEK293T cells were grown in 10-cm Petri dishes and were transiently transfected using DNA calcium phosphate coprecipitation as described (26). After 18 h, the DNA precipitate was removed by washing and the cells were left untreated for 24 h. Cells were lysed in Tris-HCl (20 mM, pH 7.5), Triton X-100 (1%), NaCl (137 mM), $MgCl_2$ (1.5 mM), EGTA (1 mM), supplemented with protease and phosphatase inhibitors. Immunoprecipitation with anti-E-tag antibodies and absorption onto protein A-linked trisacryl beads was followed by 12% SDS-polyacrylamide gel electrophoresis and blotting onto a nitrocellulose membrane. Detection of proteins was achieved with primary antibodies recognizing the E-tag or 14-3-3β. Horse-radish peroxidase-linked, anti-mouse or anti-rabbit Ig antibodies were used as secondary antibodies; detection was performed by ECL.

NF-κB reporter gene assay. HEK293T cells were grown in 24-well plates. They were transiently transfected in Optimem (using the lipofectamine procedure) with 100 ng of a pCAGGS expression vector encoding a gene of interest, 100 ng of pNFconluc, a luciferase reporter gene driven by a minimal NF-κB-responsive promoter (a kind gift of Dr. A. Israël, Institut Pasteur, Paris, France; 27), and 100 ng of a β-galactosidase (βGal)-encoding plasmid pUT651 (Cayla, Toulouse, France). After 18 h, the DNA precipitate was removed by washing and the cells were left untreated for 24 h. Following stimulation for 6 h with cytokines or phorbol ester, cells were lysed in Tris phosphate (25 mM, pH 7.8), DTT (2 mM), CDTA (2 mM), glycerol (10%) and Triton X-100 (1%). After addition of substrate buffer, finally yielding 470 μM luciferin, 270 μM coenzyme A and 530 μM ATP, luciferase activity was assayed in a Topcount microplate scintillation reader (Packard Instrument Co., Meriden, CT). βGal activity was measured, using chloramphenicol β-D-galactopyranoside (Boehringer Mannheim, Mannheim, FRG), as a substrate to normalize transfection efficiencies.

TABLE 1

A20 Interacts with 14-3-3 Proteins

Transformed yeast expression vectors		Phenotype	
		His$^+$	βGal$^+$
pAS2	pGAD424 14-3-3η	−	−
pAS2 A20	pGAD424 14-3-3η	+	+
pAS2 A20ZF$^-$	pGAD424 14-3-3η	−	−
pAS2 A20ZF$^+$	pGAD424 14-3-3η	+	+
pAS2 PAI2	pGAD424 14-3-3η	−	−
pAS2	pGAD424 14-3-3ζ	−	−
pAS2 A20	pGAD424 14-3-3ζ	+	+
pAS2 A20ZF$^-$	pGAD424 14-3-3ζ	−	−
pAS2 A20ZF$^+$	pGAD424 14-3-3ζ	+	+
pAS2 PAI2	pGAD424 14-3-3ζ	−	−

Note. Survey of histidine auxotrophy (His$^+$), tested in the presence of 5 mM 3-amino-1,2,4-triazole, and βGal activity (βGal$^+$) after cotransformation of *S. cerevisiae* strain HF7c with different yeast expression vectors. A20, A20ZF$^-$ and A20ZF$^+$ are full-length, C-terminal-deleted and N-terminal-deleted A20 cDNAs, respectively (12). Cotransformation was verified by growth on appropriate minimal synthetic media.

RESULTS AND DISCUSSION

A20 interacts with 14-3-3 proteins. Screening of a murine L929r2 fibrosarcoma cDNA library for genes which encode proteins interacting with the human zinc finger protein A20, was performed using the yeast two-hybrid system as described previously (12). 1.3×10^6 individual yeast cotransformants were screened; eleven colonies were found to express a phenotype which allowed histidine auxotrophy (His$^+$) and which was positive for βGal activity (βGal$^+$). Among the clones retained, one encoded full-length murine 14-3-3η and another murine 14-3-3ζ lacking 15 amino acids at the C-terminus. To confirm the specificity of the interactions, the pGAD424 yeast expression vectors containing the isolated 14-3-3 genes were retransformed into *S. cerevisiae* strain HF7c. This was achieved together with an empty pAS2 yeast expression vector, or with pAS2 yeast expression vectors containing full-length A20 cDNA (pAS2 A20), or with C-terminal-deleted or N-terminal-deleted A20 cDNA fragments (pAS2 A20ZF$^-$ and pAS2 A20ZF$^+$, respectively), or with plasminogen activator inhibitor-2 as irrelevant cDNA (pAS2 PAI2) (Table 1). Since histidine auxotrophy and βGal activity could only be observed in yeast colonies cotransformed with 14-3-3 cDNA and with full-length A20 cDNA or A20ZF$^+$ cDNA, we conclude that the interaction of A20 and 14-3-3 is not only specific, but is also mediated via the A20 C-terminal half which spans the domain containing the seven Cys_2/Cys_2 zinc fingers.

Next we investigated whether the protein-protein interaction between A20 and 14-3-3 observed by yeast two-hybrid screening could also be detected in mamma-

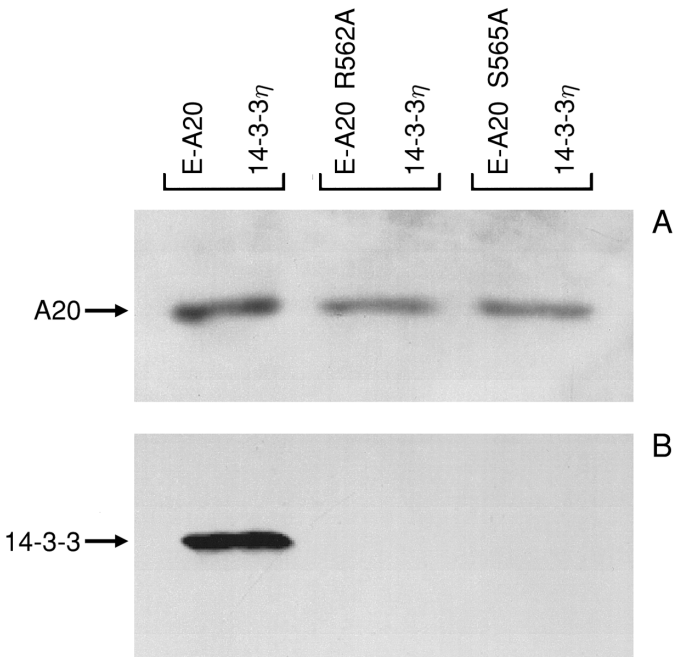

FIG. 1. Coimmunoprecipitation between A20 (muteins) and 14-3-3η in HEK293T cells. Cells were transiently transfected with pCAGGS encoding the cDNA indicated above each lane. Immunoprecipitations were performed with anti-E-tag antibody, subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using either an anti-E-tag antibody (A) or an anti-14-3-3β antibody (B).

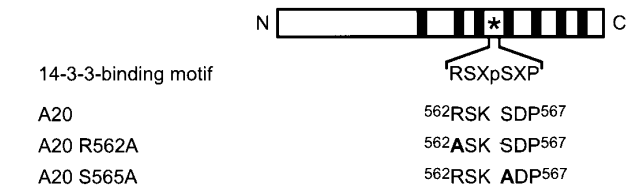

FIG. 2. Schematic representation of A20 and localization of the 14-3-3-binding motif with its mutated amino acids. Black segments represent the seven Cys_2/Cys_2 zinc fingers within A20. The asterisk indicates the 14-3-3-binding consensus motif localized between zinc fingers 3 and 4.

lian cells. To this end, we cloned the E-tagged A20 cDNA and the full-length 14-3-3η cDNA into pCAGGS, and transiently transfected both plasmids into human embryonal kidney HEK293T cells. Immunoprecipitation of the 90 kDa A20 protein with anti-E-tag antibody, followed by Western blotting with a polyclonal anti-14-3-3β antibody recognizing all members of the 14-3-3 protein family, revealed the 28-30 kDa 14-3-3η protein (Fig. 1, lane 1). We conclude that protein-protein interaction between A20 and 14-3-3 isoforms, viz. η and ζ, is also specific in higher eukaryotic cells.

Mapping of the 14-3-3-binding sequence within A20. Recent studies by Muslin et al. demonstrated that binding of 14-3-3ζ to the serine/threonine kinase raf-1 is mediated by a phosphoserine residue located within a RSXpSXP motif (21). The same consensus motif was subsequently found to be responsible for the binding of 14-3-3 to several other proteins (14). A20 contains one perfect match to this 14-3-3-binding motif, viz. the RSKSDP sequence located between zinc fingers 3 and 4 in its C-terminal half. Moreover, the RSKSDP sequence is conserved between human and murine A20 (10, 28). Metabolic labeling experiments showed that A20 is a phosphoprotein (data not shown), although the identity of the phosphorylated amino acids is not yet known. To investigate whether the RSKSDP sequence of A20 acts as a 14-3-3-binding site, we tested whether mutations within the putative 14-3-3-binding motif affected the interaction of A20 with 14-3-3 isoforms (Fig. 2). The A20 S565A mutant represents a molecule in which the serine residue possibly involved in phosphorylation and subsequent 14-3-3 binding is substituted for alanine. The second mutein, A20 R562A, represents a molecule in which the arginine residue at position −3, which is the critical determinant for phosphorylation of the aforementioned serine residue, is substituted for alanine. Using the yeast two-hybrid system, we observed that 14-3-3η and 14-3-3ζ binding to both A20 muteins is abolished, while A20 self-association is unaffected (Table 2). Loss of binding of 14-3-3η to both A20 R562A and A20 S565A was additionally confirmed in HEK293T cells by lack of coimmunoprecipitation after transient transfection of these putative interacting proteins (Fig. 1). Thus the 14-3-3-binding site within the zinc finger protein A20 can be pin-pointed to the RSKSDP sequence located between zinc fingers 3 and 4 (Fig. 2).

TABLE 2

Mapping the 14-3-3-Binding Sequence within A20

Transformed yeast expression vectors		Phenotype	
		His^+	βGal^+
pAS2 A20	pGAD424 14-3-3η	+	+
pAS2 A20 R562A	pGAD424 14-3-3η	−	−
pAS2 A20 S565A	pGAD424 14-3-3η	−	−
pAS2 A20	pGAD424 14-3-3ζ	+	+
pAS2 A20 R562A	pGAD424 14-3-3ζ	−	−
pAS2 A20 S565A	pGAD424 14-3-3ζ	−	−
pAS2 A20	pGAD424 A20	+	+
pAS2 A20 R562A	pGAD424 A20	+	+
pAS2 A20 S565A	pGAD424 A20	+	+

Note. Survey of histidine auxotrophy (His^+), tested in the presence of 5 mM 3-amino-1,2,4-triazole, and βGal activity (βGal^+) after cotransformation of *S. cerevisiae* strain HF7c with different yeast expression vectors. A20, A20 R562A and A20 S565A are full-length wild-type, R562A mutant and S565A mutant cDNAs, respectively. Cotransformation was verified by growth on appropriate minimal synthetic media.

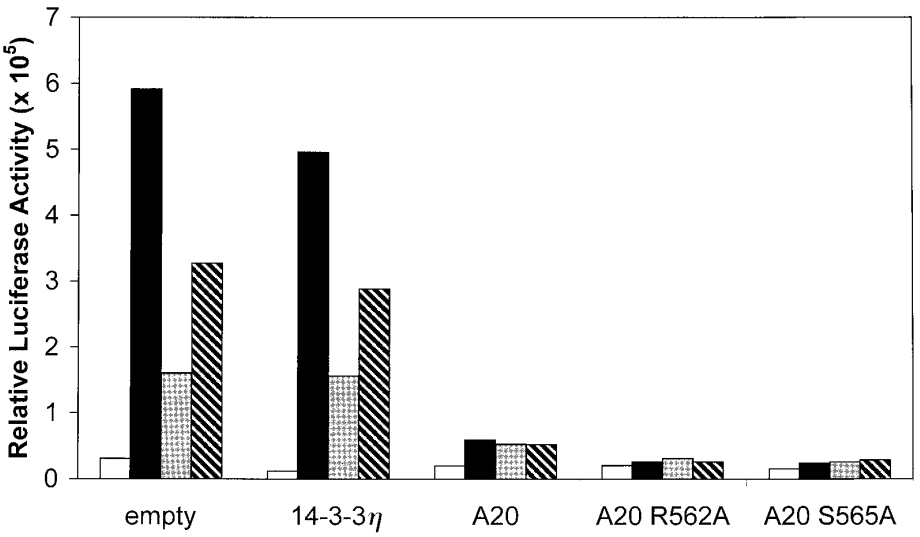

FIG. 3. Inhibition of NF-κB activation, induced by TNF, IL-1β or TPA, by overexpression of 14-3-3η or A20 (and muteins). HEK293T cells were transiently transfected with the expression vector pCAGGS carrying either no insert or inserts of 14-3-3η, A20, A20 R562A and A20 S565A cDNA. The NF-κB-responsive luciferase reporter plasmid pNFconluc and the βGal reporter plasmid pUT651 were included in each transfection. 24 h after transfection, cells were left untreated (white bars) or were stimulated for 6 h with 1,000 IU/ml TNF (black bars), 20 ng/ml IL-1β (gray bars) or 200 ng/ml TPA (hatched bars). Luciferase activity was determined in the lysates and normalized on the basis of βGal activity.

A20 inhibits NF-κB activation independently of binding to 14-3-3 proteins. An interesting feature of A20 function is inhibition of cytokine-induced or phorbol ester-induced NF-κB activation (7-9). To study whether A20 interaction with 14-3-3 proteins is crucial for this A20 function, we investigated the ability of A20 muteins, which do no longer bind 14-3-3, to inhibit NF-κB activation induced by TNF, IL-1β or TPA. To that end, HEK293T cells were transiently transfected with pNFconluc in the presence of pCAGGS expression vectors carrying 14-3-3η, A20, A20 R562A or A20 S565A cDNA inserts. We observed that NF-κB activation induced by TNF, IL-1β or TPA was equally well inhibited by coexpression of wild-type A20, A20 R562A or A20 S565A proteins (Fig. 3). In addition, NF-κB activation was not affected by coexpression of the 14-3-3η protein. These data strongly suggest that interaction with 14-3-3 proteins is not a prerequisite for this A20 function.

Despite extensive research in the past few years, the exact function of 14-3-3 proteins remains contradictory (29). The ability of 14-3-3 proteins to dimerize (18) and the resolution of their crystal structure (19, 20) suggest a role as scaffold and adaptor proteins within large complexes. This model has even been proposed for binding of 14-3-3 proteins to A20, linking A20 to c-raf (13). We have now localized the 14-3-3-binding sequence within A20 and show that binding of 14-3-3 proteins to A20 is not essential for A20-mediated inhibition of NF-κB activation by various stimuli. It might be that 14-3-3 functions as a chaperone for A20, as proposed recently (13). Other A20-associating proteins are likely to exist and might be more crucial modulators of the A20-inhibitory activity on NF-κB activation.

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