The pro-apoptotic protein death-associated protein 3 (DAP3) interacts with the glucocorticoid receptor and affects the receptor function

Sanna M. HULKKO[1]*, Hideki WAKUI*[2] and Johanna ZILLIACUS*

*Department of Medical Nutrition, Karolinska Institutet, Novum, S-141 86 Huddinge, Sweden

The yeast two-hybrid system was used to isolate cDNAs encoding proteins that interact with the glucocorticoid receptor (GR) ligand-binding domain in a ligand-dependent manner. One isolated cDNA encoded a fragment of death-associated protein 3 (DAP3), which has been implicated as a positive mediator of apoptosis. *In vitro* experiments showed that the full-length DAP3 also interacted with GR. The main interaction domain was mapped to the N-terminal region of DAP3 that had previously been shown to function in a dominant-negative fashion, protecting cells from apoptosis. Co-transfection experiments in COS-7 cells showed that DAP3 had a stimulatory effect on the ligand-induced transcriptional activation by GR and also increased the steroid-sensitivity. Furthermore, DAP3 formed a complex with several other nuclear receptors and some basic helix–loop–helix/Per–Arnt–Sim proteins, as well as with heat-shock protein 90 (hsp90) (Arnt is the aryl-hydrocarbon-receptor nuclear translocator, and Per and Sim are the *Drosophila* proteins Period and Single-minded). The results suggest that DAP3 could have an important role in GR action, possibly by modulating the cytoplasmic GR–hsp90 complex. Since glucocorticoids can induce apoptosis, the pro-apoptotic DAP3 protein may be involved in this function of GR.

Key words: COS-7, GST-pull down, nuclear receptor, yeast two-hybrid screen.

INTRODUCTION

Glucocorticoids are lipophilic hormones that affect many different functions in the body, such as development, differentiation, metabolism and the immune response [1]. The signals from glucocorticoids in target cells are mediated by the glucocorticoid receptor (GR), which is a member of the nuclear-receptor family [2]. GR has a modular structure which is composed of three distinct domains, an N-terminal domain containing a transactivation function, a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) [1]. In a glucocorticoid-free cell, GR is found in the cytoplasm in a heat-shock protein 90 (hsp90) multiprotein complex which includes hsp70, p23 and immunophilins [3]. The hsp90 complex functions as a chaperone to facilitate the folding of the GR LBD and maintaining it in a conformation competent for high-affinity ligand binding. GR interacts directly with hsp90 in the complex, dissociating upon ligand binding.

The ligand-activated GR regulates transcription of target genes, either by directly binding to the glucocorticoid response element, or by interacting with, and modulating, the activity of other transcription factors by a mechanism referred to as 'cross-talk' [1]. This may result either in activation or repression of gene transcription. Recently, many cofactor proteins that interact with the ligand-activated LBD of nuclear receptors and are required for transcriptional activation have been identified [4,5]. Some of these cofactors, such as members of the p160/SRC-1 co-activator family and the co-integrator protein CBP/p300 have been shown to have a histone acetylase function, and it has been suggested that they may enhance transcription by modulating chromatin structure [6]. Many of the cofactor proteins contain the sequence LXXLL, which is important for the interaction with the nuclear receptor and is referred to as the nuclear receptor-interacting domain (NR box) [7–9].

Knock-out mice lacking the GR are non-viable, indicating that GR is an essential protein mediating many important functions [10]. Surprisingly, the DNA-binding activity of GR is not neccessary for viability in mice [11], indicating that many of the essential glucocorticoid effects are mediated by GR using mechanisms not involving DNA-binding of the receptor. Recent results showing that GR can act by cross-talk with other transcription factors without itself binding to DNA, indicate that an important mechanism for GR action could be GR–protein interactions [1]. The specific objective in our work has been to study this alternative pathway and to identify and characterize GR-interacting proteins that are needed for the glucocorticoid effects.

In our search for novel GR-interacting proteins that modulate the activity of the receptor, we have used a yeast two-hybrid system [12] to screen a HeLa cell cDNA library utilizing ligand-activated human GR LBD as a bait. Using this method we have previously identified the 14-3-3 protein [13] that binds and regulates the function of proteins involved in cellular signalling, cell-cycle regulation and apoptosis [14]. 14-3-3 interacts with GR and stimulates the transcriptional activity of the receptor by an unknown mechanism. Here we identify and characterize an interaction between GR and death-associated protein 3 (DAP3), which was originally isolated as a protein essential for interferon-

Abbreviations used: aa, amino acids; AD, activation domain; AhR, aryl hydrocarbon receptor; AR, androgen receptor; Arnt, AhR nuclear translocator; βNF, β-naphthoflavone; DAP3, death-associated protein 3; DBD, DNA-binding domain; Dex, dexamethasone; ER, oestrogen receptor; GR, glucocorticoid receptor; GST, glutathione S-transferase; HIF1α, hypoxia-inducible factor 1α; hsp90, heat-shock protein 90; IFN-γ, interferon-γ; LBD, ligand-binding domain; NR box, nuclear receptor-interacting domain; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; RXR, retinoid-X receptor; TA, triamcinolone acetonide; TNFα, tumour necrosis factor-α; TR, thyroid receptor; bHLH/PAS, basic helix–loop–helix/Per–Arnt–Sim (Per and Sim are the *Drosophila* proteins Period and Single-minded); r, rat; h, human; DTT, dithiothreitol; Cterm, C-terminal; Nterm, N-terminal; ATP[S], adenosine 5′-[γ-thio]triphosphate.

[1] To whom correspondence should be addressed (e-mail sanna.hulkko@mednut.ki.se).

[2] Present address: Third Department of Internal Medicine, Akita University School of Medicine, 1-1-1 Hondo, Akita-shi, Akita 010-8543, Japan.

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γ (IFN-γ)-induced cell death in HeLa cells [15]. DAP3 has a functional effect on GR action, enhancing GR-activated transcription and it seems to belong to a new class of receptor-interacting proteins, possibly functioning in the cytoplasm at an early step in the GR activation pathway. Since DAP3 is a pro-apoptotic protein [15,16] and glucocorticoids are able to induce apoptosis in thymocytes [17], DAP3 may also be involved in the apoptotic function of GR.

EXPERIMENTAL

Yeast two-hybrid screen

A LexA-based yeast two-hybrid screen was performed according to the protocols from the laboratory of Dr Roger Brent (Massachusetts General Hospital, Boston, MA, U.S.A.) [12] and as described by Wakui et al. [13].

Plasmids

A cDNA encoding full-length DAP3 was isolated from a HeLa cell cDNA library (kindly provided by Dr Roger Brent) using specific PCR primers. The cDNA was inserted into the *Eco*RI/*Xho*I sites of pBKCMV (Stratagene, La Jolla, CA, U.S.A.) and pGEX4T-1 (Amersham Pharmacia Biotech, Uppsala, Sweden). To construct the N- and C-terminal deletion constructs of DAP3, specific PCR primers were used to amplify the fragments coding for amino acids (aa) 1–220 and aa 221–398 respectively. The fragments were inserted into the *Eco*RI/*Xho*I sites of pGEX4T-1. A PCR-based mutagenesis was performed in order to construct the plasmids coding for glutathione S-transferase (GST)-fused full-length DAP3, where either the NR box-like sequence (IRYLL) or the 9-aa Per–Arnt–Sim (PAS) B domain-like sequence (AYLPQELLG) was mutated by changing the leucine residues to alanine residues in respective sequences (Arnt is the aryl-hydrocarbon-receptor nuclear translocator, and Per and Sim are the *Drosophila* proteins Period and Single-minded).

The plasmids used for *in vitro* transcription–translation of full-length human GR (hGR) [13], human oestrogen receptor α (hERα) [18], human oestrogen receptor β (hERβ) [19], human thyroid receptor α (hTRα), rat retinoid-X receptor α (rRXRα) and rat peroxisome proliferator-activated receptor α (rPPARα) [20] have been described or were gifts from the following persons: Dr G. Kuiper (pBluescript-hAR), Dr K. Pettersson, (pBluescript-rPR), Dr C. Sadek (phu-hAhR and pGEM7Z-hArnt) and Dr J. McGuire (psp72-hsp90) of this Institution, and Dr C. Bradfield (pPL415-hHIF1α) and Dr C. Simon (pcDNA3-mArnt2) of the Northwestern University Medical School, Chicago, IL, U.S.A. The reporter plasmid, p19tk-luc, and GR expression plasmid, pCMV4-hGRα, used in transient transfections, have been described previously [21]. The β-galactosidase expression vector, pCMV-βgal (Stratagene), was used as an internal control in reporter gene assays. pBKCMV was used in transient transfections to keep the total amount of plasmid DNA constant.

Protein expression

[^{35}S]Methionine (Amersham Pharmacia Biotech) labelled proteins were generated using TnT® Coupled Reticulocyte Lysate system (Promega, Madison, WI, U.S.A.). GST-fusion proteins were expressed in *Escherichia coli* strain BL21(DE3)pLysS as described previously by Wakui et al. [13]. The induced proteins were purified from bacterial extracts using GSH–agarose beads (Sigma–Aldrich, St Louis, MO, U.S.A.). Sarcosyl was used to solubilize the GST–DAP3 fusion proteins which were insoluble under normal purification conditions [22].

In vitro protein–protein interaction experiments

Far-Western assay

Crude protein extracts from *E. coli* expressing GST, GST–DAP3 and GST–DAP3 (aa 1–203) were separated by SDS/PAGE and transferred to PVDF membranes (Hybond-P; Amersham Pharmacia Biotech). The membranes were incubated in hybridization buffer [25 mM Hepes/KOH (pH 7.7)/25 mM NaCl/5 mM $MgCl_2$/1 mM dithiothreitol (DTT)], containing 0.05 % (v/v) Nonidet P40 and 5 % (w/v) milk, overnight at 4 °C. After denaturation–renaturation in 6–0.187 M guanidinium chloride in hybridization buffer, the membranes were saturated at 4 °C in hybridization buffer with 0.05 % Nonidet P40 containing first 5 %, then 1 %, milk before incubation overnight at 4 °C with 250 μl *in vitro*-synthesized ^{35}S-labelled hGR in 6 ml of 20 mM Hepes/KOH (pH 7.7)/75 mM KCl/0.1 mM EDTA/2.5 mM $MgCl_2$/1 mM PMSF/1 % (w/v) milk, containing either 20 mM molybdate or 1 μM dexamethasone (Dex). The membranes were then washed five times in the same buffer and exposed for autoradiography.

GST-pull down

Purified GST-fusion proteins were bound to GSH–agarose beads at an approximate concentration of 1 μg of protein/μl of beads. A 5 ml portion of the *in vitro*-translated protein was diluted into 200 μl of ice-cold pull-down buffer [20 mM Hepes/KOH (pH 7.9)/10 % (v/v) glycerol/100 mM KCl/5 mM $MgCl_2$/0.2 mM EDTA/1 mM DTT/0.2 mM PMSF), containing 0.01 % (v/v) Nonidet P40 and 1 mg/ml BSA, and incubated at 4 °C for 2 h. A 30 μl portion of GST–protein-bound beads was incubated with 200 μl of diluted *in vitro*-translated protein at 4 °C overnight. The beads were recovered by centrifugation, washed and eluted with sample buffer. The proteins were resolved by SDS/PAGE and subjected to autoradiography. To establish that the ^{35}S-labelled protein was expressed, 10 % of the *in vitro*-translated protein input added to the incubation mix was loaded on the SDS/polyacrylamide gel. The intensity of the bands was measured by phosphoimager (Fujix BAS-200; Fuji Photo Film Co., Straubenhardt, Germany) and the relative amount of bound protein input was calculated by subtracting unspecific binding (GST) from specific binding (GST-fusion proteins).

Cell culture and transient transfections

COS-7 cells were maintained in Dulbecco's modified Eagle's medium containing sodium pyruvate and 1000 mg/litre glucose supplemented with 10 % (v/v) foetal-bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells used for reporter gene assays were seeded on to 24-well plates at a density of 2×10^4 cells/well 1 day prior to transfection. Transient transfections of cells were performed with FuGENE™-6 transfection reagent (Boehringer-Mannheim, Mannheim, Germany) according to the manufacturer's protocol. The total amount of plasmid DNA used was normalized, by the addition of an empty expression plasmid, to 0.125 μg/well. After 18 h incubation the transfection mixture was replaced with fresh medium, containing either Dex or ethanol. After 24 h the cells were harvested for enzyme assay.

Enzyme assays

To measure the luciferase and β-galactosidase activities, the cells were lysed by adding 100 μl of lysis buffer to each well. Cell lysates were assayed for luciferase activity by using GenGlow-1000 luminescence kit (BioOrbit, Turku, Finland), and for β-

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galactosidase activity using Galacto-Light Plus® chemiluminescence kit (Tropix Inc., Bedford, MA, U.S.A.). The enzyme activities were measured with an Anthos LucyI microplate luminometer (Anthos Labtec Instruments, Salzburg, Austria). All experiments were carried out in triplicate, repeated at least three times, and luciferase activity was normalized to β-galactosidase activity.

Immunoprecipitation with anti-hsp90 antibodies

The immunoprecipitation experiments were performed as described by McGuire et al. [23].

RESULTS

A C-terminal fragment of DAP3 interacts specifically and ligand-dependently with GR LBD in the yeast two-hybrid system

In a LexA-based yeast two-hybrid screen, using a HeLa cell cDNA library, we isolated cDNA fragments that encoded proteins interacting with human GR LBD (aa 485–777) in a ligand-dependent manner. The ligand used in these experiments was triamcinolone acetonide (TA). Sequence analysis revealed that one of the clones encoded a short C-terminal portion (aa 324–398) of DAP3, a protein which was originally isolated as a factor essential for IFN-γ-induced apoptosis in HeLa cells [15] (Figure 1). The interaction of the C-terminal DAP3 fragment with the ligand-activated GR LBD was subsequently verified. The plasmid expressing the isolated fragment of DAP3 fused to the B42 activation domain (B42 AD), or the control plasmid expressing only the B42 AD, was retransformed into either the original yeast strain, expressing LexA-fused GR LBD, or into other strains expressing LexA-fused GR DBD or the bicoid protein. The interaction of the DAP3 fragment with GR LBD was specific, since only the strain co-expressing LexA-fused GR LBD and the B42 AD-fused fragment of DAP3 grew on Leu$^-$ plates (results not shown). Furthermore, the interaction was ligand-dependent, as cell growth was only observed on the medium containing the synthetic glucocorticoid TA (results not shown). To study the interaction more in detail, the level of *lacZ*-reporter-gene expression in extracts prepared from the yeast strains was determined, using a β-galactosidase liquid assay. The strain co-expressing the LexA-fused GR LBD and B42 AD-fused fragment of DAP3 (C-DAP3) showed strong β-galactosidase activity in the presence of TA (Figure 2A). In accordance with the results seen with the Leu$^-$ plates, the DAP3 fragment did not interact with GR DBD or bicoid protein (Figure 2A). In addition, the interaction of the C-terminal fragment of DAP3 with GR LBD was tested in the presence of various synthetic glucocorticoids. The two GR agonists tested, Dex and TA, strongly induced the protein–protein interaction, but the antagonist, RU486, had a smaller effect (Figure 2B).

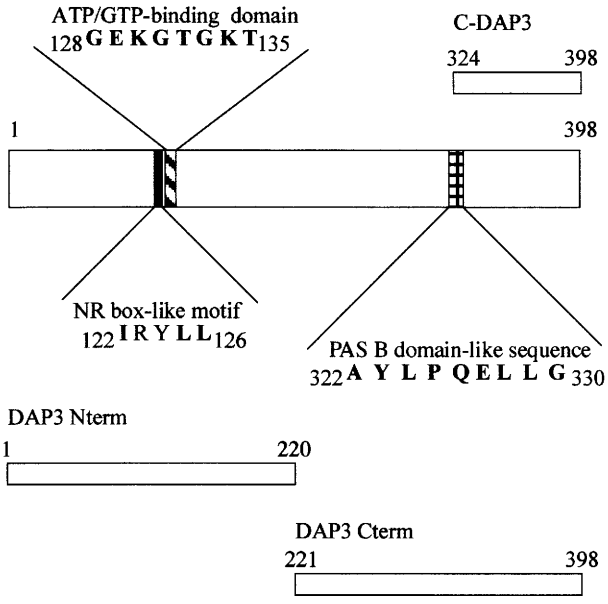

Figure 1 Schematic representation of DAP3

Location and amino acid sequences for the NR-box-like-motif (black box), the putative ATP/GTP-binding domain (hatched box) and the 9-aa PAS-B-domain-like sequence (grid box) are shown. The characteristic amino acids for each motif are written in **bold** letters. The C-terminal fragment of DAP3 (C-DAP3) isolated as a GR-LBD-interacting clone in the yeast two-hybrid screen, as well as the N-terminal (DAP3 Nterm) and C-terminal (DAP3 Cterm) fragments used in *in vitro* GST-pull-down assays are shown.

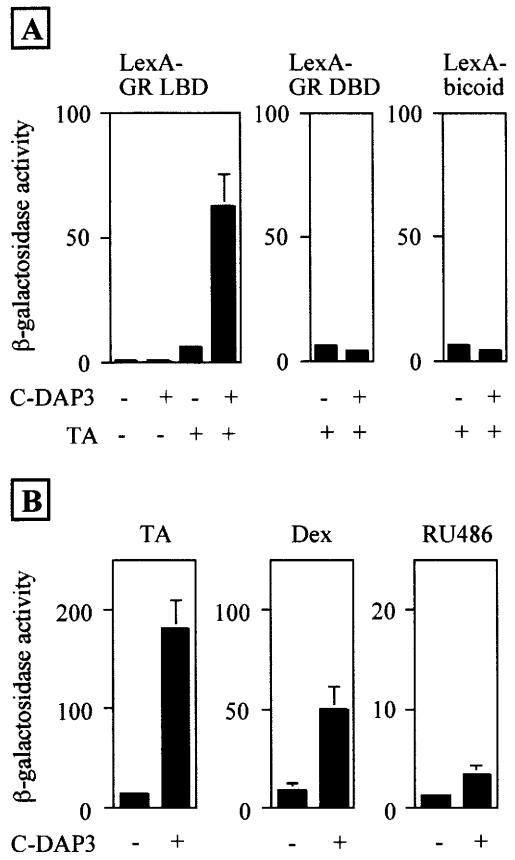

Figure 2 The interaction between the C-terminal fragment of DAP3 and the GR LBD is ligand-dependent and specific in a yeast two-hybrid system

(**A**) Plasmids expressing B42 activation domain (B42 AD) or B42 AD-fused C-terminal fragment of DAP3 (C-DAP3) were introduced into EGY188 yeast strains containing *lacZ* reporter genes under the control of LexA-binding sites and plasmids expressing LexA-fused GR LBD, GR DBD or the bicoid protein. Transformants were grown in a selective medium in the presence or absence of 10 μM TA. (**B**)The EGY188 yeast strain expressing LexA-fused GR LBD was transformed with expression plasmids for either B42 AD or B42 AD-fused C-terminal fragment of DAP3 (C-DAP3). Transformants were grown in a selective medium containing 10 μM TA, 50 μM Dex or 50 μM RU486. β-Galactosidase activity in cell extracts of liquid yeast cultures was assayed 5 h after treatment with glucocorticoids. Each extract was measured in triplicate and the values represent the means $\pm$ S.D.

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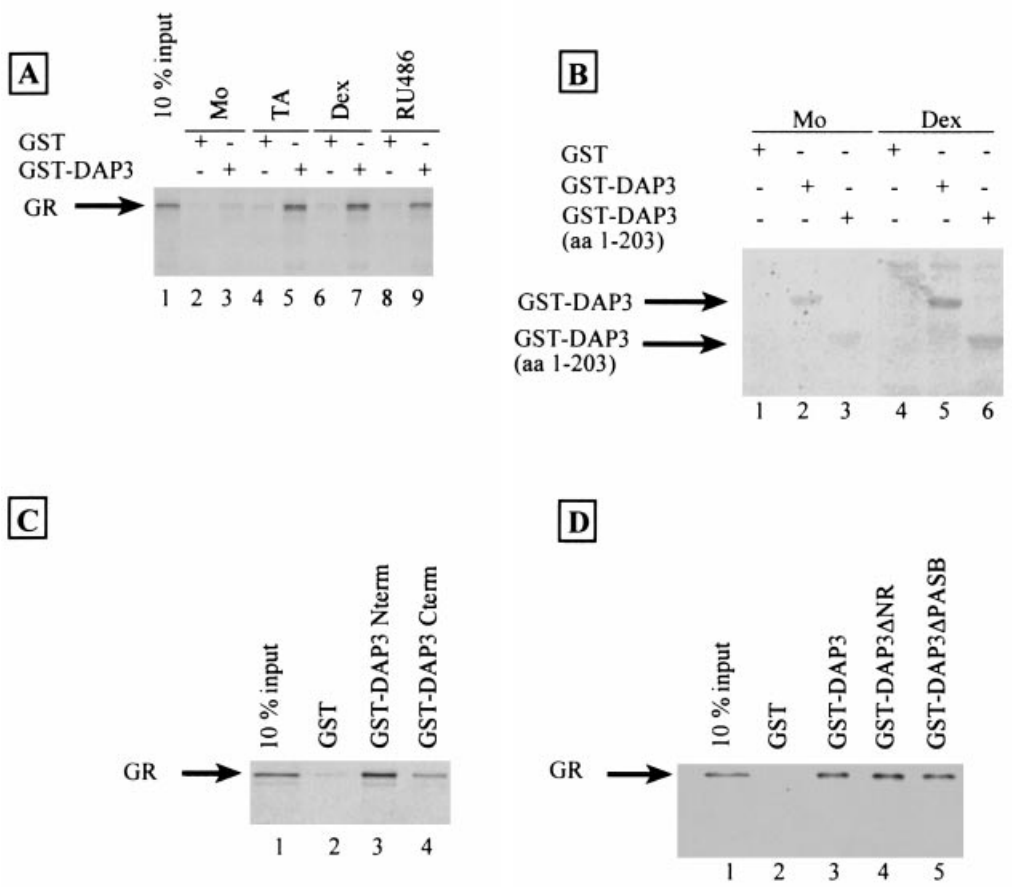

Figure 3 ***In vitro*** **interaction of DAP3 with GR**

(**A**) GST-pull-down assay. ^{35}S-labelled full-length GR was generated by *in vitro* translation and incubated with GSH–agarose beads bound by GST (lanes 2, 4, 6 and 8) or GST–DAP3 (lanes 3, 5, 7 and 9) in the presence of 20 mM molybdate (Mo, lanes 2 and 3), 0.2 μM TA (lanes 4 and 5), 1 μM Dex (lanes 6 and 7) or 1 μM RU486 (lanes 8 and 9) as indicated. The eluted proteins from washed beads were analysed by SDS/PAGE and revealed by autoradiography. The input (lane 1) always represents 10% of the amount of labelled protein used. (**B**) Far-Western assay. ^{35}S-labelled GR was incubated with GST (lanes 1 and 4), GST–DAP3 (lanes 2 and 5) or GST–DAP3(aa 1–203) (lanes 3 and 6) immobilized on membranes in the presence of 20 mM molybdate (lanes 1–3) or 1 μM Dex (lanes 4–6) as indicated. The membranes were washed and exposed for autoradiography. (**C**) and (**D**) GST-pull-down assay. ^{35}S-labelled GR was incubated with GST or the indicated GST–DAP3 constructs bound to GSH–agarose beads in the presence of 1 μM Dex. DAP3 deletion constructs (DAP3 Nterm and DAP3 Cterm) contain aa 1–220 and 221–398 respectively. GST–DAP3$\triangle$NR has a mutated NR-box-like motif and GST—DAP3$\triangle$PASB a mutated 9-aa PAS-B-domain-like sequence.

Full-length DAP3 interacts with GR *in vitro*

A full-length cDNA of DAP3 was isolated from a HeLa-cell cDNA library and the cDNA was used to investigate the *in vitro* interaction of DAP3 with the unliganded, hsp90-associated GR and the ligand-activated, hsp90-dissociated GR. *In vitro*-translated GR forms a complex with hsp90 in rabbit reticulocyte lysate, and the complex is stabilized by molybdate [24]. GST-pull-down experiments showed a weak interaction between the *in vitro*-translated GR and GST–DAP3 in the presence of molybdate (Figure 3A, lane 3). The strength of the interaction was clearly increased in the presence of TA or Dex (Figure 3A, lanes 5 and 7). Analysis of the experiment by PhosphoImager indicated that GST–DAP3 bound four times more liganded GR than unliganded, hsp90-associated receptor. Treatment of GR with the antagonist RU486 resulted in a decreased level of interaction between the receptor and GST–DAP3 compared with the interaction in the presence of agonist (Figure 3A, lane 9). The *in vitro* interaction between GR and full-length DAP3 could also be seen in a Far-Western assay (Figure 3B). In accordance with the results from the GST-pull-down experiment, the interaction of full-length DAP3 with GR was weak in the presence of molybdate (Figure 3B, lane 2), and was clearly increased when Dex was added to the incubation mixture (Figure 3B, lane 5).

The major GR-interaction surface of DAP3 is mapped to the N-terminal region

To identify the major interaction site of DAP3 for GR, GST–DAP3 fusion proteins containing either the N-terminal (DAP3 Nterm, aa 1–220) or the C-terminal (DAP3 Cterm, aa 221–398) fragments of the protein were generated (Figure 1). The fusion proteins were used in GST-pull-down experiments. As shown in Figure 3(C), lane 3, there was a strong interaction between GR and DAP3 Nterm, but the binding of GR to DAP3 Cterm was only moderate (Figure 3C, lane 4). The PhosphoImager analysis indicated that DAP3 Nterm binds five times more GR than DAP3 Cterm. In addition, it was observed in the Far-Western assay that another N-terminal fragment of DAP3, DAP3$^{1–203}$, interacted with liganded GR (Figure 3B, lane 6). The interaction of DAP3$^{1–203}$ with GR was weak in the presence of molybdate (Figure 3B, lane 3).

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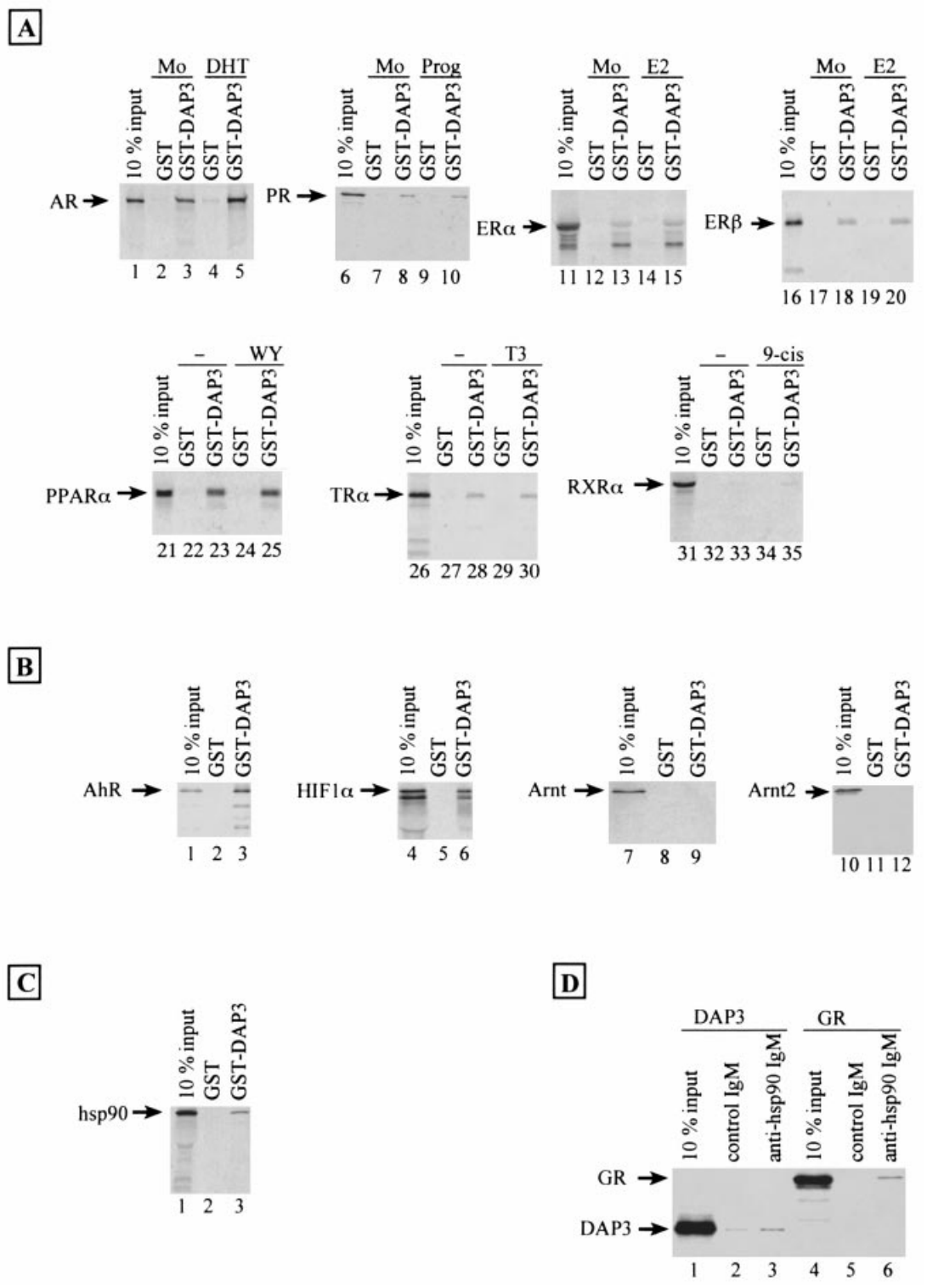

Figure 4 DAP3 is not a GR-specific interactor

AR, PR, ERα, ERβ, PPARα, TRα and RXRα (**A**), AhR, HIF1α, Arnt and Arnt2 (**B**) and hsp90 (**C**) were *in vitro* translated in the presence of [^{35}S]methionine. The *in vitro* interaction of the proteins with GSH–agarose-bound GST or GST–DAP3 was analysed using GST-pull-down assays as described in the legend to Figure 3(A). The interaction of AR, PR, ERα and ERβ was studied in the presence of 20 mM molybdate (Mo) or 1 μM of the appropriate ligand, i.e. dihydrotestosterone (DHT), progesterone (Prog) and 17β-oestradiol (E2) as indicated. The interaction of TRα, RXRα and PPARα was studied in the absence (—) or presence of 10 μM of their respective ligands, i.e. WY-14,643 (WY), L-tri-iodothyronine (T3) and 9-*cis*-retinoic acid (9-cis) as indicated. The interaction of AhR was studied in the presence of 100 μM βNF. The interaction of HIF1α, Arnt, Arnt2 and hsp90 was examined in the absence of ligand. (**D**) *In vitro* translated and [^{35}S]methionine-labelled DAP3 or GR was co-immunoprecipitated with hsp90 present in rabbit reticulocyte lysate using either control IgM (lanes 2 and 5) or anti-hsp90 IgM (lanes 3 and 6). The immunoprecipitate was analysed by SDS/PAGE and subjected to autoradiography. The input (lanes 1 and 4) represents 10% of the translated protein added to the incubation mixture.

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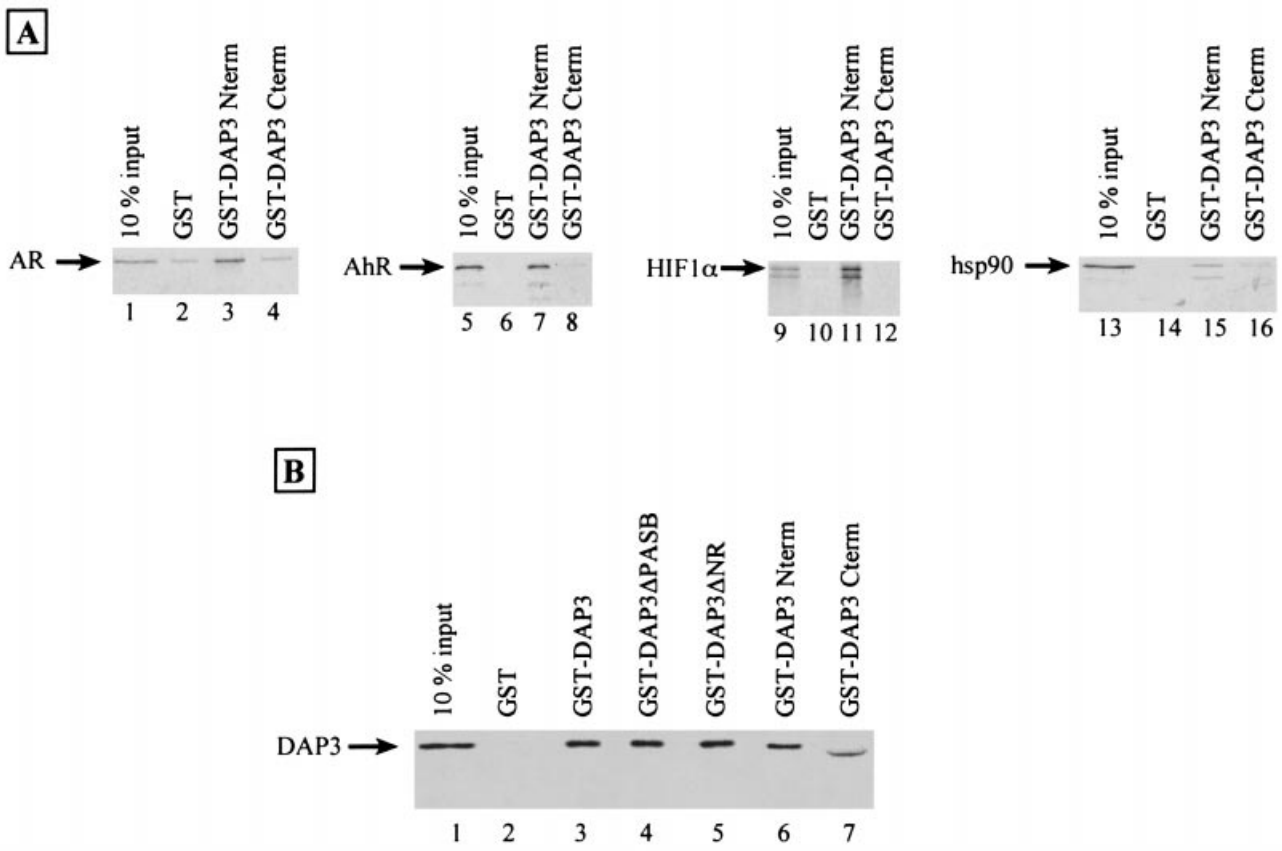

Figure 5 The major protein interaction site of DAPs and its ability to dimerize

(**A**) The major protein interaction surface of DAP3 is mapped to the N-terminal region. ^{35}S-labelled AR, AhR, HIF1α and hsp90 were generated by *in vitro* translation and incubated with GST or the indicated GST–DAP3 constructs bound to GSH–agarose beads. The interaction of AR and AhR was studied in the presence of 100 nM DHT and 100 μM βNF respectively. (**B**) DAP3 is able to dimerize *in vitro*. The ability of the *in vitro* translated and ^{35}S-labelled proteins to interact with GST or indicated GST–DAP3 constructs bound to GSH–agarose beads was analysed using a GST-pull-down assay as described in the legend to Figure 3(A). The DAP3 constructs are as described in the legend to Figures 3(C) and (D). The specific DAP3 band in Figure 5(B), lane 7, is smeary, since it is running at the same position as the GST-fusion protein.

The N-terminal portion of DAP3 contains the sequence IRYLL, which is closely related to the consensus NR box known to be important for cofactor–nuclear receptor interactions [7–9] (Figure 1). The C-terminal portion of DAP3 contains a 9-aa sequence, AYLPQELLG, which is also found in the PAS B domain of BMAL1/MOP3, and with 2-aa and 3-aa divergency in Arnt [AhR (aryl-hydrocarbon receptor) nuclear translocator] and Arnt2, which are all members of the basic helix–loop–helix/Per–Arnt–Sim (bHLH/PAS) protein family [25–28] (Figure 1). The PAS B domain is involved in protein–protein interactions between different bHLH/PAS proteins [29,30], as well as between the AhR and hsp90 [31]. These motifs were mutated individually in the context of the full-length GST–DAP3 by replacing the leucine residues with alanine residues. The sequence changes decrease the hydrophobicity of the interaction surface, but are not expected to destroy the putative α-helical structure of the motifs. The GST-fusion proteins generated (GST–DAP3$\triangle$NR and GST–DAP3$\triangle$PASB) were used in GST-pull-down assays. As shown in Figure 3(D) (compare lanes 3 and 4), we observed a slight increase in GST–DAP3$\leftrightarrow$GR interaction when the NR box-like motif was mutated. The increase in binding was only 1.5-fold, as determined by PhosphoImager analysis. The mutation of the 9-aa PAS B domain-like sequence did not appear to affect the binding of GST–DAP3 to GR (Figure 3D; compare lanes 3 and 5).

DAP3 is not specific for GR

A range of nuclear receptors were tested for their putative interactions with DAP3 using the GST-pull-down assay. The steroid receptors known to form a complex with hsp90 were tested either in the presence of molybdate in order to stabilize the receptor–hsp90 complex, or in the presence of a specific ligand. The other receptors were treated with or without a specific ligand. As shown in Figure 4(A), GST–DAP3 bound to all receptors tested, except retinoid-X receptor α (RXRα), albeit with various affinities. GR, androgen receptor (AR) and PPARα interacted most strongly with DAP3 (Figure 3A, lanes 5 and 7, and 4A, lanes 5 and 25). Although molybdate treatment reduced the binding of GR to DAP3 (Figure 3A, compare lane 3 with lanes 5, 7 and 9), it did not markedly affect the DAP3 interactions with the other receptors (Figure 4A). In addition, DAP3 interacted with PPARα and TRα in a ligand-independent manner (Figure 4A).

The interaction of DAP3 with members of the bHLH/PAS protein family, as well as hsp90, was also studied. As demon-

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strated in Figure 4(B), lane 3, GST–DAP3 interacted strongly with AhR in the presence of the ligand β-naphthoflavone (βNF). GST–DAP3 was also able to interact with hypoxia-inducible factor-1 α (HIF1α) (Figure 4B, lane 6), but was unable to bind either Arnt or Arnt2 (Figure 4B, lanes 9 and 12 respectively). When the interaction between AhR and DAP3 was tested in the presence of molybdate, the interaction was reduced approx. 2-fold (results not shown). A weak interaction between GST–DAP3 and *in vitro*-translated hsp90 was observed (Figure 4C, lane 3). The interaction between DAP3 and hsp90 was verified by a co-immunoprecipitation assay in which *in vitro*-translated DAP3 was co-immunoprecipitated with hsp90 present in reticulocyte lysate using a hsp90 specific antibody (Figure 4D, lane 3). Co-immunoprecipitation of *in vitro*-translated GR with hsp90, using the same antibody, was used as a positive control (Figure 4D, lane 6).

The N-terminal region of DAP3 is the major protein–protein interaction site

The interaction of AR, AhR, HIF1α and hsp90 with GST-fused DAP3 Nterm and DAP3 Cterm was studied by GST-pull-down assays. All the proteins tested interacted preferentially with DAP3 Nterm (Figure 5A, lanes 3, 7, 11 and 15). Only hsp90 showed an interaction with DAP3 Cterm, although this interaction was very weak (Figure 5A, lane 16). In addition, we determined whether the mutation of the NR box-like or 9-aa PAS B domain-like sequences in the context of the full-length protein would affect the interaction of DAP3 with AR, AhR or hsp90. As with GR, no clear differences between the interactions of GST–DAP3 and GST–DAP3ΔNR or GST–DAP3ΔPASB with AR, AhR or hsp90 were observed (results not shown). A putative ATP/GTP-binding domain has been located in the N-terminal portion of DAP3 [15] (Figure 1). The effects of ATP and the hydrolysis-deficient analogue adenosine 5′-[γ-thio]triphosphate (ATP[S]), on the protein interactions of DAP3 were determined in a GST-pull-down assay as well. The nucleotides slightly decreased DAP3 interaction with both GR and hsp90 (results not shown).

DAP3 is able to dimerize *in vitro*

The ability of DAP3 to dimerize has been investigated using the GST-pull down assay. Our results show that *in vitro* translated DAP3 was able to bind to GST-fused DAP3 (Figure 5B, lane 3). The dimerization was not limited to the full-length proteins, as the truncated forms GST–DAP3 Nterm and GST–DAP3 Cterm were also able to interact with the full-length DAP3 (Figure 5B, lanes 6 and 7 respectively). Neither the mutation of the NR box-like or the 9-aa PAS B domain-like sequence appeared to affect dimer formation with the intact *in vitro* translated DAP3 (Figure 5B, lanes 4 and 5 respectively).

DAP3 enhances the transcriptional activity of GR

In transient co-transfection assays in COS-7 cells, DAP3 enhanced the expression of a glucocorticoid-response-element-driven luciferase reporter gene (p19tk-luc) by the ligand-activated GR in a dose-dependent manner. At the highest amount of DAP3 tested, Dex-induced transactivation by GR was enhanced 9-fold by DAP3 (Figure 6A). DAP3 did not affect the reporter-gene activity in the absence of GR (results not shown). To investigate whether DAP3 also affected the steroid-sensitivity, a dose–response curve was determined (Figure 6B). The concentration of Dex required to induce half-maximal activity of the luciferase reporter gene was reduced in cells transfected with

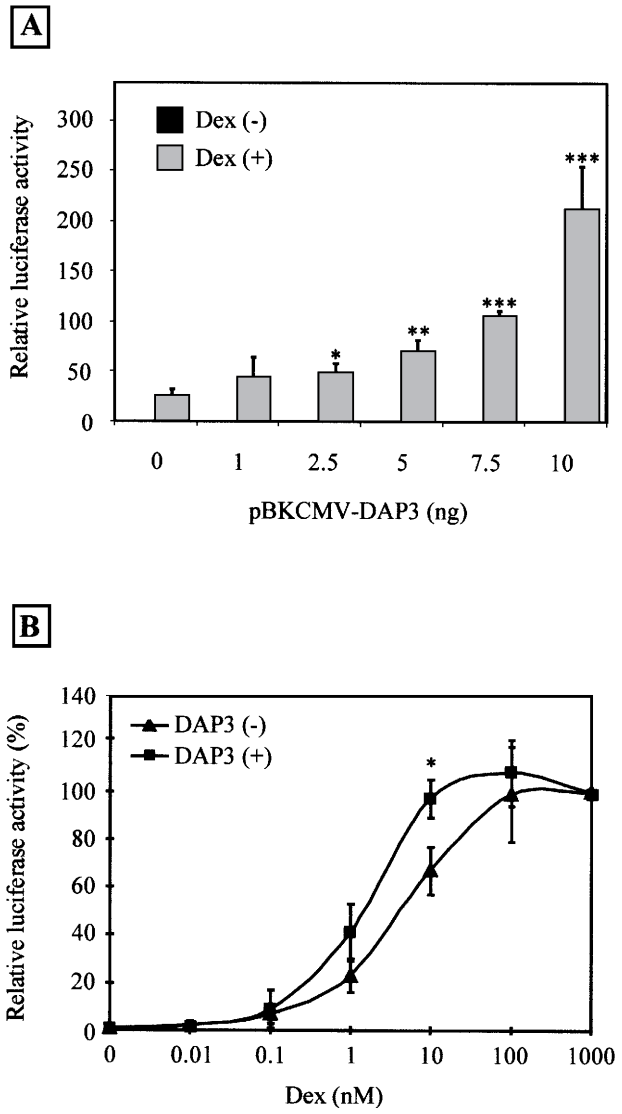

Figure 6 Stimulatory effect of DAP3 on the transcriptional activity of ligand-activated GR

(**A**) COS-7 cells were transiently transfected with 100 ng of p19tk-luc and 5 ng of pCMV-βgal reporter plasmids, and 10 ng of pCMV4-hGRα expression plasmid, as well as increasing amount of pBKCMV-DAP3 expression plasmid. The total amount of pBKCMV plasmid was kept constant by using the empty plasmid. The cells were treated with or without 1 μM Dex. The values without Dex are not visible, since they are all below one. (**B**) Dose–response curve of Dex for GR transactivation in the absence and presence of DAP3 expression plasmid. The cells were transfected with the reporter plasmids and GR expression plasmid as in (**A**), and with 10 ng of pBKCMV-DAP3 expression plasmid or an empty pBKCMV plasmid. The cells were treated with or without the indicated amount of Dex. The relative luciferase activity is presented as percentage activation, where the value of 100% represents the activity in the presence of 1 μM Dex. In all assays, the GR-regulated luciferase-reporter-gene activity was related to the activity of the constitutively expressed β-galactosidase activity in cells treated with or without Dex for 24 h, and the results in each case represent mean values $\pm$ S.D. obtained from three independent transfections. A two-tailed two-sample equal-variance *t*-test was used to analyse the statistical significance of the results: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. In (**A**) the *P* values were calculated by comparing the relative luciferase values of the Dex-treated cells transfected with pBKCMV-DAP3 to similarly treated pBKCMV-transfected cells.

DAP3. A colorimetric 'MTT' [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay [32] showed that introduction of DAP3 expression plasmid into COS-7 cells did not

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reduce either the viability or growth of the cells (results not shown).

DISCUSSION

Recently, many studies have suggested that transactivation of gene expression by nuclear receptors is modulated by receptor interactions with other proteins, such as co-activators and co-repressors of transcription, as well as co-integrators of diverse signal-transduction pathways [4,5]. These proteins act at a step subsequent to receptor binding to the DNA by modulating the chromatin structure or the transcriptional machinery [4,5]. However, only a few proteins that function at other parts of the receptor pathway have been described [3,13]. We have applied a LexA-based yeast two-hybrid system to identify proteins that interact with the ligand-activated human GR LBD. One of the proteins identified, the DAP3 protein, seems to belong to a distinct class of functional receptor-interacting proteins separate from the well-characterized co-activator proteins.

In *in vitro* assays a weak interaction between GR and DAP3 was observed in the presence of molybdate, which is known to stabilize the GR–hsp90 chaperone complex formed in rabbit reticulocyte lysate [24]. The hsp90 complex functions by maintaining GR in an inactive form until ligand binding, as well as stabilizing GR in a conformation that has high affinity for the ligand. However, the binding of DAP3 to GR was markedly induced upon agonist treatment of the receptor and to a lesser extent by the GR antagonist RU486. These *in vitro* results indicate that prior dissociation from the hsp90 multiprotein complex and an agonist-induced conformational change of GR is necessary for a strong interaction with DAP3.

DAP3 is not a GR-specific interactor and was shown to interact with several other nuclear receptors as well as the bHLH/PAS proteins AhR and HIF1α. However, it did not bind to RXRα, Arnt or Arnt2, and the strength of the DAP3–protein interactions varied, which indicates some degree of interaction specificity. The binding of DAP3 to other nuclear receptors than GR was ligand-independent and not inhibited by molybdate, which is probably due to a weaker complex formation between these receptors and hsp90 [3]. The bHLH/PAS proteins AhR and HIF1α are also reported to interact with hsp90 [33,34] and in the presence of molybdate the binding of AhR to DAP3 was impaired, although not as much as for GR. Interestingly, most of the proteins that have shown a strong interaction with DAP3 (i.e GR, AR, AhR and HIF1α), also bind to hsp90. In addition, DAP3 itself showed a weak interaction with hsp90.

The mutation of either the NR box-like sequence or the 9-aa PAS B domain-like sequence did not clearly affect the *in vitro* protein interactions of DAP3. However, these sequences may have importance in fine tuning and regulation of the many different DAP3–protein interactions and the function of DAP3. By changing the leucine residues to alanine residues in respective sequences, the hydrophobicity of the motifs is reduced, but the putative α-helical stucture is not expected to be disrupted. To be able to observe more considerable changes in interactions and to study the role of these sequences more in detail, one may have to perform mutations that disrupt the structure of the motifs, although such mutations could also lead to altered folding of the full-length protein. The putative ATP/GTP-binding domain of DAP3 has been shown to be essential for the pro-apoptotic effect of DAP3, as well as for the dominant-negative effect of DAP3 Nterm [16]. Both ATP and hydrolysis-deficient ATP analogue ATP[S] only slightly impaired the *in vitro* interactions of DAP3. This indicates that hydrolysis of ATP is not required for the negative effect observed on the protein interactions of DAP3 and that the nucleotide-binding domain of DAP3 probably has another functional role not related to protein binding.

In transient transfections of COS-7 cells the transactivation activity of GR was stimulated by increasing amounts of DAP3. The increase in reporter gene activity was not due to the pro-apoptotic effect of DAP3, because no reduction in cell viability or growth was observed. DAP3 also increased the steroid-sensitivity of GR transactivation, suggesting that DAP3 could modulate the ligand activation of GR.

The mechanism of DAP3 action may be related to the fact that DAP3 is able to bind hsp90. Although DAP3 has not so far been identified as a protein in the hsp90 multiprotein complex, it is possible that DAP3 could transiently interact with the complex and function at the step of release of GR from the hsp90 complex.

DAP3 has previously been identified as a protein with a pro-apoptotic function. DAP3 functions as a positive mediator of IFN-γ [15], Fas- and TNF-α [16]-induced cell death, although the mechanism for this effect is still unknown. An N-terminal fragment of DAP3 (aa 1–230) has been shown to function in a dominant-negative fashion, protecting cells from apoptosis [16]. In the yeast two-hybrid screen, it was a short C-terminal portion of DAP3 that was identified as an interaction partner for GR LBD. However, *in vitro* GST-pull-down experiments indicated that the major interaction site of DAP3 with GR was the N-terminal region of DAP3 (aa 1–220). The other DAP3-interacting proteins also bound preferentially the N-terminal construct of DAP3. This suggests that the dominant-negative effect on apoptosis exerted by the N-terminal fragment of DAP3 could result from DAP3 binding to some cellular proteins involved in the apoptotic pathway. Furthermore, we have shown that DAP3 can form a homodimer, and the N-terminal region is enough for dimer formation with the full-length protein. It is thus possible that DAP3 functions as a dimer and that the dominant-negative effect is due to the formation of an inactive DAP3 dimer between the N-terminal DAP3 fragment and the full-length protein. Glucocorticoids are strong inducers of apoptosis in thymocytes, but the molecular mechanism is still largely unknown [17]. It is thus interesting to speculate that DAP3 could be involved in the pro-apoptotic effect of GR.

In summary, these findings suggest that DAP3 is an important protein modulating GR function, which may contribute to glucocorticoid-induced apoptosis. In addition, DAP3 may modulate the activity of a number of hsp90-binding proteins, including nuclear receptors and bHLH/PAS proteins.

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