A RAS recruitment screen identifies ZKSCAN4 as a glucocorticoid receptor-interacting protein

Karin Ecker¹, Andreas Lorenz¹, Frank Wolf¹, Christian Ploner¹, Günther Böck², Tod Duncan³, Stephan Geley¹ and Arno Helmborg¹

¹Biocenter, Division of Molecular Pathophysiology, Innsbruck Medical University, Fritz Pregl Strasse 3, A 6020 Innsbruck, Austria

²Biocenter, Division of Developmental Immunology, Innsbruck Medical University, Fritz Pregl Strasse 3, A 6020 Innsbruck, Austria

³Department of Biology, University of Colorado, Denver, 1200 Larimer Street, NC 3016 E, Denver, Colorado 80204, USA

(Correspondence should be addressed to A Helmborg; Email: arno.helmborg@i-med.ac.at)

Abstract

To search for proteins interacting with the glucocorticoid receptor, we adapted Aronheim's reverse RAS recruitment system relying on the *Saccharomyces cerevisiae* mutant *cdc25-2*, which has a temperature-dependent defect in its RAS signaling pathway driving proliferation. The full-length human glucocorticoid receptor (NR3C1, isoform- α) was attached to the yeast plasma membrane in either of two orientations and used as bait to screen a HeLa cell cDNA library. Library proteins were fused to constitutively active, soluble human RAS, complementing the defective yeast pathway in case of bait-prey interaction. Screening of 800 000 clones resulted in the isolation of 21 proteins, 8 of which were followed up to evaluate interaction with the receptor in human cell lines. One of these candidates, the SCAN- and KRAB-domain-containing zinc finger protein 307 (ZKSCAN4) was co-precipitated with the receptor when both proteins were overexpressed in HEK293 cells. Rabbit antisera against ZKSCAN4 were raised, affinity purified, and used to immunoprecipitate endogenous ZKSCAN4 from Hct116 cells, resulting in co-precipitation of endogenous glucocorticoid receptor. Overexpressed ZKSCAN4 was found to co-localize in granular nuclear structures with the activated glucocorticoid receptor and partially with chromatin regions characterized by histone H3 mono-methylated on lysine 4 (H3K4me1). Overexpressed ZKSCAN4 had no effect on an episomal glucocorticoid receptor-driven reporter plasmid. By contrast, ZKSCAN4 markedly reduced glucocorticoid induction of the mouse mammary tumor virus-promoter-driven reporter gene when this was chromosomally integrated, arguing for a chromatin-dependent inhibition of glucocorticoid receptor-mediated transactivation.

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Introduction

Glucocorticoids, a class of steroid hormones released from the adrenal gland, play a key role in the regulation of metabolism, immune responses, and inflammatory reactions (Sapolsky *et al.* 2000, Necela & Cidlowski 2004). Synthetic equivalents are widely used as drugs to treat allergic, autoimmune, and certain malignant diseases (Schimmer & Parker 2005). Glucocorticoids enter the cell by free diffusion across the lipid bilayer of the cell membrane. In the cytoplasm, they interact with the glucocorticoid receptor (GR), which, in the absence of ligand, is bound to a large, dynamic multi-protein chaperone complex. This complex includes heat shock protein molecules Hsp90 and, temporarily, Hsp70 as well as an immunophilin component and is essential to keep the receptor in a high affinity state for hormone binding (Dittmar *et al.* 1997, Pratt & Toft 1997). Upon association with its ligand, the ubiquitously expressed receptor translocates to the nucleus

where it acts as a transcriptional regulator, interacting either directly with DNA or with other proteins involved in transcriptional regulation.

Interactions with other proteins have been extensively investigated in two functional settings: first, for the cytoplasmic multi-protein complex containing the unliganded receptor (Pratt *et al.* 2006) and secondly, for the liganded, nuclear receptor in the context of transcriptional regulation (Necela & Cidlowski 2004, Copik *et al.* 2006). Much less effort has been devoted to investigating potential effects of the GR without pertinence to transcription, meaning important interactors might have been missed so far (Stellato 2004). Sengupta *et al.* described ligand-dependent interaction of the GR with p53 and MDM2, building up a trimeric complex in the cytoplasm. Interaction of these proteins leads to cytoplasmic sequestration and proteasome-dependent degradation of both GR and TP53. (Sengupta *et al.* 2000, Sengupta & Wasyluk 2001). In one recent systematic study, the GR was immunoprecipitated from cytosolic rat

liver extracts, and co-immunoprecipitated proteins were separated on 2D-gels, resulting in hundreds of spots, of which 34 were identified by mass spectrometry (Hedman *et al.* 2006). However, these candidates were not followed up. To also allow the detection of cytoplasmic interaction partners, we used the GR as a cytoplasmic, membrane-bound bait to perform an unbiased search for further interacting proteins.

A typical tool to search for protein–protein interactions is the yeast two-hybrid (Y2H) system. In its classical form, the system makes use of the reconstitution of transcription factor activity via the interaction of two proteins (the ‘prey’ and the ‘bait’), one fused to the activation domain of a transcription factor and the other fused to the corresponding DNA-binding domain, to facilitate expression of reporter genes (Fields & Song 1989). This system has the inherent limitation that proteins containing transactivating domains cannot be used as bait, as they would directly activate the expression of reporter genes. Consequently, only parts of the GR excluding the large transactivation domains have so far been used in Y2H-screens (Gottlicher *et al.* 1996, Hittelman *et al.* 1999, Yang *et al.* 2000). To overcome this limitation, Aronheim and co-workers developed alternative cytoplasmic systems, one of which, the reverse RAS recruitment system (rRRS), we adapted, using the GR as bait (Aronheim 2001, Hubsman *et al.* 2001); instead of a transcription factor activity, the temperature-dependently defective RAS-signaling pathway is reconstituted in the *Saccharomyces cerevisiae* mutant strain *cdc25-2*. This strain (Petitjean *et al.* 1990) harbors a temperature-sensitive mutation (E1328K) in CDC25, rendering it inactive and unable to activate RAS at the restrictive temperature of 37 °C. The GR α -isoform was used as bait, attached to the yeast plasma membrane by an N-terminal myristoylation or a C-terminal farnesylation sequence. Prey proteins expressed from a HeLa cDNA library were fused to constitutively active human (h)RAS lacking its farnesylation domain (RAS(61) Δ F; Broder *et al.* 1998). The RAS mutant is able to substitute for the defective yeast protein if brought into the proximity of the membrane via the interaction of the cDNA-encoded moiety with the GR. Thus, growth signaling at the restrictive temperature is reconstituted, resulting in colony formation. In contrast to other systems, this procedure allows the identification of proteins interacting with the full-length GR under cytoplasmic conditions of a eukaryotic cell and results in the isolation of a defined span of cDNA as a starting point for follow-up experiments.

Materials and methods

Expression plasmids for the Y2H system

GR-expression plasmids pKECKhGR-myr and pKECKhGR-CAAX are based on the vector pADNS

(*LEU2*; *ampR*) (Colicelli *et al.* 1989). A multiple cloning site containing a HindIII as well as a NotI restriction site was inserted and the ADH1-promoter was exchanged by the Met25-promoter derived from plasmid pMet25 (Mumberg *et al.* 1994) using standard cloning techniques. The ampicillin resistance gene was replaced by a kanamycin resistance gene by recombination in strain EL250 (Lee *et al.* 2001) as described (Yu *et al.* 2000), forming the plasmid pKECK. Of the different human splice variants and isoforms (Yudt & Cidlowski 2001, Presul *et al.* 2007), the classical GR α (A) open reading frame (ORF) was amplified by standard PCR technique from plasmid pEF Δ T-hGRwt (Riml *et al.* 2004) in frame with either a C-terminal CAAX-sequence (leading to post-translational farnesylation) encoded in the reverse primer or with an N-terminal myristoylation (Myr)-sequence encoded in the forward primer and subsequently ligated into the HindIII/NotI restricted vector pKECK.

The yeast RAS-fusion cDNA library (*URA3*; *ampR*) from proliferating HeLa cells was generated as previously described (Broder *et al.* 1998). Fusion proteins were expressed under control of the GAL1-promoter. Constitutively active, soluble hRAS (RAS(61) Δ F) is fused N-terminally to the protein fragments encoded by oligo-dT-primed HeLa cDNA. The library had a complexity of 800-000 clones before a single round of amplification.

Expression plasmids for human cell lines

cDNAs for candidate genes were obtained from RZPD (clone IRAUp696B0365D for ZKSCAN4). Coding sequences for full-length ZKSCAN4, the different truncated mutants, and GR α were amplified by standard PCR technology and cloned into plasmid pDONR207 (Invitrogen), followed by further subcloning into Flag- or cyan fluorescent protein (CFP)-destination vectors using GATEWAY technology (Invitrogen, Carlsbad, CA, USA), resulting in plasmids pE-FLAG-ZKSCAN4 or pE-CFP-ZKSCAN4 respectively. The GR was expressed without tag (pE-GR) or, for the purpose of microscopy studies, in frame with a yellow fluorescent variant (YFP)-tag at the N-terminus (pE-YFP-GR). Plasmid phPR-SG5, based on vector pSG5 (Stratagene, La Jolla, CA, USA), contains the full-length cDNA of the human progesterone receptor (PR) under control of the SV40-promoter and was a kind gift of Dr Wolfgang Doppler, Biocenter, Innsbruck Medical University.

Plasmids for bacterial overexpression

To obtain a 6xHis-tagged version of ZKSCAN4, the coding sequence was subcloned into vector pDEST17 (Invitrogen) using GATEWAY technology (pE-6xHis).

For the purpose of antibody affinity purification, an N-terminal fragment of the ORF (ZKSCAN4_(AA 1–194)) was subcloned into a destination vector encoding N-terminal GST (pE-GST-ZKSCAN4).

Yeast media, growth conditions and transfections

S. cerevisiae strain *cdc25-2* has been previously described (Petitjean *et al.* 1990). Yeast cells were grown at 25 or 37 °C on glucose or galactose minimal medium containing 0.17% Difco yeast nitrogen base without amino acids, 0.5% NH₄SO₄, the appropriate added amino acids and 2% glucose or a mixture of 3% galactose, 2% raffinose, and 1% glycerol. For plates, 4% of agar was added. Methionine was supplemented at a concentration of 200 µg/ml to repress methionine-promoter-dependent transcription. Transfections were performed as previously described (Aronheim *et al.* 1994).

Cell culture, transfections, and lysis conditions

U2OS, Hct116, and HEK-293T cells were maintained in DMEM (Cambrex, East Rutherford, NJ, USA), CEM-C7H2 and Jurkat cells were cultured in RPMI medium (Cambrex), both supplemented with 10% FCS (PAA laboratories GmbH, Linz, Austria), 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.3 mg/ml glutamine (Cambrex). DNA and siRNA transient transfections were performed using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. For inducing the GR for nuclear translocation, dexamethasone (dex) was added to the medium at a final concentration of 10^{−7} M. For total lysates, cells were washed with PBS, harvested from the culture dish by a rubber policeman, centrifuged at 1000 r.p.m. for 5 min, and lysed in NP-40 based lysis buffer (20 mM Tris-HCl (pH 8), 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) for 1 h at 4 °C. CEM-C7H2 cells were stably transfected with a ZKSCAN4-expression construct by retroviral transfection.

Generation of Jurkat GR MMTV-VNP cells

The lentiviral pHR-MMTV-VNP construct was generated by inserting the mouse mammary tumor virus LTR-promoter (MMTV) from pMMTV-Luc (Doppler *et al.* 2001) into the MluI-SnaBI sites of pHR-MCS-VNP. The latter plasmid was constructed by PCR amplifying venus-NLS-Pest (VNP (Nagoshi *et al.* 2004)) using primers 5'-tatagaattcggatccacgcgtgtttaactacgtagccacatcgtgagcaagggc-3' (sense) and 5'-tatagcggcgcccatatgactagttacacattgatcc-3' (antisense). The purified fragment, containing a primer-generated multiple cloning site and VNP, was inserted into the EcoRI/NotI-sites of

the lentiviral plasmid pHR-SIN-CSGW-ΔNot (Demaision *et al.* 2002), thereby replacing the spleen focus-forming virus (SFFV)-eGFP cassette, to generate pHR-MCS-VNP.

Generation of MMTV-VNP expressing cells

Preparation of lentiviral supernatant was described elsewhere (Ploner *et al.* 2008). In brief, 3 µg of the lentiviral construct together with 2 µg pCMV 8.91 and 2 µg pMD-G plasmids were transfected into the HEK-293T packaging cell line. Lentiviral supernatant was harvested 48 and 72 h after transfection, sterile filtered, supplemented with 4 µg/µl polybrene (Sigma St Louis, MO, USA) and 5 × 10⁵ Jurkat-GR cells (Helmberg *et al.* 1995) were infected twice with 500 µl lentiviral supernatant.

Generation of ZKSCAN4 overexpressing Jurkat GR MMTV-VNP cells

The lentiviral constitutive expression plasmid pHR-SFFV-ZKSCAN4-ires-Puro was generated by recombining pENTR-307-ZKSCAN4 with the 'destination vector' pHR-SFFV-Dest-ires-Puro (Ploner *et al.* in preparation), facilitating the simultaneous expression of ZKSCAN4 and, for selection, the puromycin resistance gene under control of the SFFV-promoter. The bulk of MMTV-VNP expressing Jurkat GR cells were subsequently infected with the lentiviral construct coding for ZKSCAN4, following the same procedure as described above.

Co-immunoprecipitation experiments

For co-immunoprecipitation (co-IP) experiments with overexpressed proteins, 24 µg DNA were used to transfect HEK-293T and Hct116 cells in a 10 cm dish at 90% confluency with plasmids pE-FLAG-ZKSCAN4 and pE-GR. 48 h after transfection, cells were lysed in a HEPES-based lysis buffer (0.2% Triton X-100; 50 mM HEPES (pH 7.5); 150 mM NaCl; 10% glycerol; protease inhibitor cocktail from Roche; 50 mM NaF; 5 mM NaOV) for 1 h at 4 °C followed by incubation of 1 mg lysate with 10 µg FLAG-antibody (F3165; Sigma-Aldrich), 5 µg ZKSCAN4-antibody, or 5 µg GR-antibody (sc-1003; Santa-Cruz Biotechnology, Santa Cruz, CA, USA) respectively. After incubation for 1 h at 4 °C, lysates were centrifuged for 15 min at 13 000 r.p.m. to eliminate potential antibody-antigen super-complexes. Supernatant was further incubated with protein G-agarose beads (E3403; Sigma-Aldrich) for 1 h at 4 °C followed by four washing steps with the lysis buffer. The co-IP samples were subjected to SDS-PAGE and western blotting with FLAG- or GR-antibody respectively. As a negative control, lysates were incubated with

protein G-Sepharose beads only or with isotype controls.

ZKSCAN4-antibody production and affinity purification

A 6xHis-tagged version of ZKSCAN4 was expressed in *E. coli* strain BL21(DE3)pLysS from plasmid pE-6xHis-ZKSCAN4 upon induction with 2 mM IPTG and purified by binding to Ni-NTA agarose (1018244; Qiagen). Purified fusion protein together with Freund's adjuvant was used to immunize two rabbits in 4-week intervals over a total time period of 6 months. The first immunization was performed with complete (F-5991; Sigma), further immunizations with incomplete Freund's adjuvant (F-5506; Sigma). To affinity purify the antibody, a GST-tagged version of an N-terminal fragment of the protein (GST-ZKSCAN4_(AA 1–194)) was bound to a Glutathione-Sepharose matrix (17-0756-01; Amersham) and covalently cross-linked using 40 mM dimethyl pimelinediimide (80490; Fluka) in 0.1 M borate buffer (pH 9). After application of rabbit antiserum to the column, bound ZKSCAN4-antibody was eluted with 0.2 M glycine (pH 2.0) and buffered immediately. The resulting affinity-purified antibody-eluate was unstable and only usable for about 10 d at 4 °C, and was therefore frozen in small aliquots upon purification.

Fluorescence microscopy and immunostaining

Microscopy was performed with an Axiovert 200M live cell imaging instrument and a Zeiss Axiovert 100 confocal microscope with LSM510 laserhead. For live cell microscopy, home-made glass-bottomed culture dishes were used and cells were monitored at 37 °C and 5% CO₂.

For immunostaining experiments, cells were fixated with methanol for 4 min at –20 °C. Antibodies for immunostaining: Y12 (Acris; DM3198); mono-methyl H3K4 (Abcam; ab8895); tri-methyl H3K4 (Abcam; ab8580); PML (Santa Cruz Biotechnology; Sc-966); our self-made affinity purified rabbit anti-ZKSCAN4; Alexa Fluor anti-rabbit 488 (Invitrogen; A11008); Alexa Fluor anti-rabbit 568 (Invitrogen; A10042); Alexa Fluor anti-mouse 594 (Invitrogen; A11062).

Site-directed mutagenesis

To mutate the two arginines in the sequence of CFP-ZKSCAN4_(AA 193–365) to alanines (R339A, R340A), site-directed mutagenesis was performed by PCR with Pfu-polymerase using forward and reverse primers containing the appropriate nucleotides under standard PCR conditions. DpnI restriction was performed to

disrupt the old methylated DNA template. Competent *E. coli* cells were transformed with the PCR product and isolated plasmids (CFP-ZKSCAN4_(AA 193–365)ΔNLS) were checked by sequencing.

Immunoblotting

Lysates were boiled in Laemmli buffer (250 mM Tris-HCl, (pH 6.8); 20% glycerol; 6% SDS; 0.1% bromophenol blue; 1.43 M β-mercaptoethanol) for 5 min, separated by SDS-PAGE on a 12.5% gel and electrophoretically transferred onto a nitrocellulose membrane. Membranes were blocked with 5% defatted milk in TBS-T for 1 h at 4 °C followed by incubation with the primary antibody, diluted in TBS-T, over night at 4 °C. After three washing steps, blots were incubated with secondary antibodies coupled to horse radish peroxidase and specific bands were detected using Immobilon HRP detection reagent (Millipore, Bedford, MA, USA). Antibodies used for immunoblotting: FLAG (F3165; Sigma-Aldrich, St Louis, MO, USA); GR (sc-1003; Santa-Cruz Biotechnology); affinity purified rabbit anti-ZKSCAN4; GAPDH (ab9484; Abcam, Cambridge, UK).

Results

Screening for GR-interacting proteins by the reverse RAS recruitment system

Two different yeast *cdc25-2* strains were generated by transfection with either GR-expression vector pKECK-hGR-myr or pKECK-hGR-CAAX. In these cells, the full-length GRα (bait) was expressed as fusion protein with an N-terminal myristoylation or a C-terminal farnesylation (CAAX) sequence. Membrane anchorage of the receptor by these post-translational lipid modifications was verified by western blot analysis of membrane and soluble fractions of cell lysates. The GR was expressed under control of the methionine-repressible Met25-promoter. High methionine concentrations strongly reduced the expression of the bait, while lack of methionine in the medium allowed expression, as shown in Fig. 1A for the GR-CAAX fusion protein.

A flow diagram of the screening procedure is shown in Fig. 1B. Strain *cdc25-2* expressing either one of the GR fusions was transfected with the Gall-promoter-driven RAS-fusion HeLa cDNA library. After incubation on glucose minimal medium at the permissive temperature of 25 °C, library colonies were replica plated onto two galactose-containing plates differing in methionine concentrations (0 µg/ml or 200 µg/ml) and incubated at the restrictive temperature of 37 °C. Down-regulation of GR-expression by methionine allowed testing for

Figure 1 Screening procedure. (A) Expression of GR-CAAX in *cdc25-2*. GR-expression was repressed by an addition of methionine to the growth medium (lanes 3 and 4). A high level of protein was found in total cell lysates (lane 1; T) and in the membrane fraction of cell lysates (lane 2; M). No signal could be detected in the soluble fraction (lane 5; S). Lower panel, Coomassie staining. (B) Flowchart. Step I: after 3 days of growth on glucose medium at 25 °C, colonies were replica plated onto galactose plates. Colonies that showed preferred growth on the plate lacking methionine after 3 days of incubation at 37 °C (indicated by an arrow) were picked, transferred to glucose plates, cultivated at 25 °C, and tested again for methionine-dependent difference in growth ability on galactose/37 °C in a first verification step (positive clones: arrows in step II). Step III: Positive clones were transfected into strain *cdc25-2* lacking bait. If this clone (10 independent colonies) was unable to grow at the restrictive temperature, the cDNA insert was sequenced.

false positive library proteins that attach to the membrane *per se*. Replica plating on a glucose plate allowed identification of potential revertants. After incubation at the restrictive temperature, colonies showing preferred growth on the plate lacking methionine were picked as candidates and subjected to a reconfirmation step. In a last verification procedure, the corresponding prey plasmid was isolated and transfected into *cdc25-2* devoid of the GR-expression construct to once more exclude potential bait-independent growth. Screening was performed in the presence and absence of 10^{-6} M deoxycorticosterone, a glucocorticoid that, in contrast to dex, has been shown to be active in yeast (Garabedian & Yamamoto 1992), but no candidates were observed that unequivocally mediated growth only in the presence of hormone.

Of 800 000 colonies screened, 70 primary candidates were finally isolated and sequenced. Sequencing allowed to eliminate 70% of these candidates from further consideration. Some of the eliminated candidates showed fusions of the cDNA to RAS in another than the ORF, frequently resulting in an artificial short, hydrophobic protein fragment. A subset of positive, but characteristically very small colonies was found to only contain the poly-A tail of cDNA, resulting in a polylysine peptide with obviously some non-specific affinity to the GR. Other candidate fragments derived from ORF within the 3' untranslated regions of mRNAs. Although potentially interesting, these candidates were not further pursued, as were a few proteins with established extracellular roles. The remaining 21 candidates are listed in Table 1.

One of this small number of candidates, HSPA5, has already been described to interact with the GR *in vitro* (Hutchison *et al.* 1996), serving as a positive control, and another, TRAP1, is a close mitochondrial relative of Hsp90, the main chaperone in the cytoplasmic GR-complex. TRAP1 binding to the GR would thus not be surprising, but was not further verified.

A novel binding partner for the GR: ZKSCAN4

Based on the information available in the literature and databases, we selected eight candidate proteins for the next screening step, consisting of expressing full-length FLAG-tagged versions of the respective proteins in human cells and assaying for potential co-IP with the GR. In this step, interaction was confirmed for the 62 kDa (545 AA) zinc finger protein 307 (ZKSCAN4; *Homo sapiens* zinc finger with KRAB and SCAN domains 4/ZKSCAN4). HEK-293T cells were co-transfected with expression vectors encoding GR and N-terminally FLAG-tagged zinc finger protein (FLAG-ZKSCAN4_(full length)). Immunoprecipitation of the receptor from total cell lysates resulted in co-precipitation of ZKSCAN4 as detected with the FLAG-antibody (Fig. 2).

Table 1 Potential glucocorticoid receptor (GR)-interacting proteins, identified using the reverse RAS recruitment system (rRRS)

		Localization
Protein		
AHS1	Activator of 90 kDa heat shock protein ATPase homolog 1	C
CYPA	Cyclophilin A	C
DBI (isoform 3)	Endozepine	C, M
YBX1	DNA-binding protein B	C, M
DYNLRB1	Dynein light chain of the robl/LC7 group	C
EEF1A1	Elongation factor 1- α	C
EEF1G	Elongation factor 1- γ	C
GNL3	Nucleostemin	NO
HSPA5	Heat shock 70 kDa protein 5	ER
TRAP1	Heat shock protein 75	M, C
KRT10	Keratin 10	C, N
MRPL4	Ribosomal protein L4	C
RPL37	Ribosomal protein L37	C
LonP1	Lon peptidase 1	M
MPRIIP	Myosin phosphatase-Rho interacting protein	C
Rho	Nascent polypeptide-associated complex α -poly-peptide	C, N
PNAS 101	NB4 apoptosis-related protein	?
PSMB6	Proteasome subunit β -type 6 precursor	C
RAN	RAS-related nuclear protein	C, N
THMB10	Thymosin β 10	C, N
ZKSCAN4	Zinc finger protein 307	N

C, cytoplasm; ER, endoplasmic reticulum; M, mitochondrium; N, nucleus; NO, nucleolus.

To examine interaction of the GR with the endogenous zinc finger protein, we generated rabbit antisera against the full-length protein and subsequently affinity-purified specific antibodies using the GST-coupled N-terminal SCAN domain. Using this antibody, we tested the expression of ZKSCAN4 in five human cell lines by western blot analysis, finding higher levels of protein in HeLa, Hct116, and CEM-C7H2 (Fig. 3A; lanes 2, 4, and 6) than in U2OS and HEK-293T cells, where ZKSCAN4 was hardly detectable (not shown). As control, lysates of HeLa and Hct116 cells, transfected with plasmid pE-FLAG-ZKSCAN4 (lanes 1 and 3), or lysates of stably

ZKSCAN4-transfected CEM-C7H2 (lane 5) cells were used. Using the affinity-purified antibody to immunostain endogenous ZKSCAN4 in fixated HeLa cells demonstrated an exclusively nuclear, granular distribution (Fig. 3B). Specificity of staining was controlled for by co-transfected siRNA: siRNA against ZKSCAN4 abrogated staining, while siRNA against unrelated luciferase did not.

Immunoprecipitation of endogenous ZKSCAN4 from Hct116 cell lysates using the affinity-purified antibody resulted in co-precipitation of the endogenous GR (Fig. 4A), confirming the interaction between the two proteins at physiological expression levels. Co-precipitation of endogenous proteins could not be demonstrated in HeLa or CEM-C7H2 cells. While ZKSCAN4 was identified as a GR-interacting protein, it conceivably might also interact with other nuclear receptors. To test for potential interaction between the PR and ZKSCAN4, we co-transfected HEK-293T cells with plasmids leading to the overexpression of these two proteins. Immunoprecipitation of ZKSCAN4 did not result in co-immunoprecipitation of PR (Fig. 4B).

As with other candidate proteins, we compared the spatial distribution of ZKSCAN4 to that of the GR by live cell wide-field and confocal fluorescence microscopy. In U2OS osteosarcoma cells, ZKSCAN4 was expressed as a hybrid protein with an N-terminal CFP. The GR was fused to the YFP. CFP-ZKSCAN4 was localized

Figure 2 Overexpressed Flag-ZKSCAN4 co-immunoprecipitates with GR. HEK-293T cells were transiently transfected with expression vectors encoding FLAG-ZKSCAN4 and GR. Cells were lysed and equivalent amounts of lysates were immunoprecipitated with antibody against FLAG or GR or isotype control antibody as indicated. Immunoprecipitates were separated by SDS-PAGE and probed by immunoblotting using anti-FLAG. Input represented 4% of the lysate used in one IP reaction.

Figure 3 Characterization of an affinity-purified rabbit antibody raised against ZKSCAN4. (A) Detection of endogenous and overexpressed ZKSCAN4 in human cell line immunoblots. Whole cell lysates of HeLa, Hct116, and CEMC7H2 cells were prepared either from untransfected cells (–) or from cells transiently transfected with FLAG-ZKSCAN4 or stably transfected with untagged ZKSCAN4 (CEM-C7H2) as indicated (+), and sequentially probed with anti-ZKSCAN4 and anti-GAPDH. Note that in the FLAG-ZKSCAN4-transfected lanes, part of the over-expressed protein is not FLAG-tagged due to usage of the normal in-frame start codon. Slightly higher than the FLAG-ZKSCAN4, an unspecific band (*) is recognized by the ZKSCAN4-antibody. (B) Detection of endogenous ZKSCAN4 by immunocytochemistry. HeLa cells were transiently transfected with luciferase- (as control) or ZKSCAN4-specific siRNA, fixed and stained with affinity purified rabbit antibody against ZKSCAN4, and fluorescence and phase contrast images were taken.

exclusively in the nucleus in either of two patterns: in a granular pattern in the nucleoplasm as seen with the endogenous protein, sometimes forming a ring-like structure around the nucleoli (Fig. 5A), or homogeneously distributed in the nucleoli (Fig. 5B). While the GR was cytoplasmic in U2OS cells washed with serum-free medium, dex-addition shifted the receptor to the nucleus within 5 min where it first showed a slightly granulated pattern followed by homogeneous nuclear distribution, as shown in a time lapse acquisition in Fig. 5C. Concomitant overexpression of CFP-ZKSCAN4 led to a redistribution of YFP-GR to the granular nucleoplasmic structures of ZKSCAN4 (confocal image, Fig. 5E). Live cell microscopy showed that the GR was recruited to the speckled CFP-ZKSCAN4 pattern immediately after nuclear translocation, until the patterns became virtually identical (Fig. 5D; note the difference in YFP-GR distribution to Fig. 5C).

Figure 4 (A) Endogenous GR is co-immunoprecipitated with endogenous ZKSCAN4. Hct116 cells were treated with dex for 12 h and lysed. Equivalent amounts of lysates were immunoprecipitated with antibody against GR, ZKSCAN4, or isotype control antibody as indicated. Immunoprecipitates were separated by SDS-PAGE and probed by immunoblotting using GR-antibody. (B) ZKSCAN4 does not co-immunoprecipitate the progesterone receptor. HEK-293T cells were transiently transfected with expression vectors encoding FLAG-ZKSCAN4 and PR. Cells were lysed and equivalent amounts of lysates were immunoprecipitated with antibody against ZKSCAN4 or isotype control as indicated. Immunoprecipitates were separated by SDS-PAGE and probed by immunoblotting using anti-PR and anti-FLAG antibodies. Input represented 4% of the lysate used in one IP reaction.

Identification of a ZKSCAN4 nuclear localization signal

ZKSCAN4 contains three conserved domains: an N-terminal SCAN, or leucine-rich domain, the name-giving zinc finger domain at the C-terminus comprising seven C2H2 zinc fingers, and a middle KRAB-(Kruppel-associated box)-A domain. To narrow down the domains of ZKSCAN4 essential for co-localization with the GR, we constructed expression vectors for each individual domain and combinations thereof (Fig. 6A), expressed in frame with an N-terminal CFP, and monitored their subcellular localization relative to the GR (Fig. 6B). The two N-terminal domain fragments CFP-ZKSCAN4_(AA 1–294) and CFP-ZKSCAN4_(AA 1–194) lost their ability for nuclear translocation and localized entirely in the cytoplasm. The small CFP-ZKSCAN4_(AA 193–294) was homogeneously distributed, probably due to passive diffusion of the 41 kDa protein through the nuclear pore complex. All the other fragments resided in the nucleus. Only CFP-ZKSCAN4_(AA 193–545),

Figure 5 Subnuclear localization of ZKSCAN4 and the GR. (A, B) U2OS cells were transiently transfected with an expression vector encoding CFP–ZKSCAN4, stained with Hoechst 33342, and fluorescence images were taken as indicated. (C) U2OS cells were transiently transfected with an expression vector encoding YFP–GR, treated with 10^{-7} M dex, and filmed. Panels show fluorescence images taken every minute. (D) U2OS cells were transiently transfected with expression vectors encoding CFP–ZKSCAN4 and YFP–GR, treated with 10^{-7} M dex and filmed. Panels show fluorescence images taken every 10 min. (E) U2OS cells were transiently transfected with expression vectors encoding CFP–ZKSCAN4 and YFP–GR, treated with 10^{-7} M dex, and confocal fluorescence images were taken after 15 h.

consisting of KRAB plus zinc finger domain, co-localized with the induced GR in the typical granular pattern as shown for the full-length protein in Fig. 5D and E. The C-terminal fragment CFP–ZKSCAN4_(AA 366–545) showed nucleolar localization, indicating the presence of a nucleolar targeting signal sequence in the zinc finger domain.

The observed difference in cellular location between the two fragments containing only the KRAB-domain (CFP–ZKSCAN4_(AA 193–294) and CFP–ZKSCAN4_(AA 193–365)) suggested the presence of a nuclear localization signal (nls) between amino acids 295 and 365 as the

longer fragment was exclusively nuclear. This area contains a KHRR sequence that might function as nuclear targeting sequence. By exchanging the two arginines to alanines by site-directed mutagenesis in the 49 kDa fusion protein CFP–ZKSCAN4_(AA 193–365) (R339A; R340A), the fragment lost its exclusively nuclear localization, proving the nls-function of amino acids KHRR (Fig. 6C). By contrast, exchange of the two amino acids in the full-length construct did not prevent nuclear localization (data not shown). Therefore, the presence of additional nuclear localization signals in the zinc finger domain seems likely.

Figure 6 Cellular localization of ZKSCAN4 domain constructs and identification of a nuclear localization sequence. (A) Schematic of the ZKSCAN4-parts of CFP–ZKSCAN4 fusion proteins. (B) U2OS cells were transiently transfected with expression vectors encoding the indicated individual CFP–ZKSCAN4 domain construct and YFP–GR, treated with 10^{-7} M dex and fluorescence images were taken after 12 h. (C) U2OS cells were transiently transfected with an expression vector encoding CFP–ZKSCAN4_{(AA 193–365)ΔNLS}, and fluorescence and phase contrast images were taken after 40 h.

Relationship of ZKSCAN4 to known subnuclear structures

To assess whether ZKSCAN4 might be part of known subnuclear structures, we compared the distribution of ZKSCAN4 with the distributions of informative proteins. No overlap was found with the Y12 protein, which is typical for the classical ‘speckles’ involved in splicing (Lamond & Spector 2003). Also, there was no overlap with PML-bodies, which form a distinct speckled pattern and have been reported to be involved in transcriptional

regulation (Fig. 7B; Zhong *et al.* 2000), or with nuclear regions enriched for histone H3 tri-methylated on lysine 9, which is typical for regions that have been silenced by conversion to heterochromatin (Fig. 7C). However, we found partial co-localization of ZKSCAN4 with nuclear regions enriched in histone H3 mono-methylated on lysine 4 (H3K4me1) which may be poised for transcriptional activation (Schneider *et al.* 2004) (Fig. 7D). By contrast, very little co-localization was observed for the tri-methylated form H3K4me3 (Fig. 7E).

Figure 7 Localization of CFP-ZKSCAN4 in relation to well-characterized subnuclear structures. U2OS cells were transiently transfected with expression vectors encoding CFP-ZKSCAN4, fixed and stained with (A) Y12-antibody or (B) antibodies against PML, (C) histone H3-trimethyl-K9, (D) histone H3-monomethyl-K4, or (E) histone H3-trimethyl-K4.

Chromatin-dependent inhibition of GR-mediated transactivation

A considerable number of proteins that are structurally similar to ZKSCAN4 exist in the human genome, and members of this group have been implicated in splicing and transcriptional regulation (Urrutia 2003). As ZKSCAN4 did not co-localize with Y12, a role in splicing seemed improbable. Another established role for similar proteins, which was also recently claimed for

ZKSCAN4, is in transcriptional repression by several mechanisms (Huang *et al.* 1999, Hennemann *et al.* 2003, Li *et al.* 2007). To assess a potential effect on glucocorticoid-dependent transcription, we tested the effect of ZKSCAN4 on a luciferase reporter construct driven by the glucocorticoid-responsive MMTV-promoter in a transient transfection assay. Transiently co-transfecting COS-7 cells with ZKSCAN4 and GR-expression constructs, as well as the MMTV-luciferase reporter, we did not find any significant effect of ZKSCAN4 on promoter activity of this episomal vector in the presence or absence of glucocorticoids (data not shown). However, some aspects of MMTV regulation are chromatin dependent and can only be assessed when the MMTV-LTR is integrated into chromosomal DNA (Deroo & Archer 2001). We therefore stably integrated a MMTV-promoter-driven reporter construct into a GR-overexpressing Jurkat subline (Helmberg *et al.* 1995). Treatment of this bulk culture with dex leads to the induction of VNP (Venus-NLS-PEST; Nagoshi *et al.* 2004), which is an exclusively nuclear derivative of YFP with a short half-life of ~20 min, to closely reflect time-dependent cellular mRNA levels. Dex-dependent activity of the chromosomally integrated MMTV-promoter can thus be monitored by FACS analysis (Fig. 8A). We then infected cells of this culture with a selectable lentiviral construct resulting in constitutive overexpression of ZKSCAN4. In cells overexpressing ZKSCAN4, induction of VNP by treatment with dex for 14 h was markedly reduced compared with control cells (Fig. 8B): relative fluorescence intensity of ZKSCAN4 overexpressing cells was 45 vs 133 in VNP-positive control cells (window M1 in Fig. 8B).

Discussion

In this study, we used the full-length human GR to identify novel interacting proteins by applying the yeast reverse rRRS. By screening ~800 000 independent colonies, we identified 21 potential candidates. One of these, HSPA5, had been shown previously to bind to the GR *in vitro* (Hutchison *et al.* 1996), demonstrating that the system is in fact able to identify binding partners. We chose the reverse rRRS for two reasons. First, it allowed us to use the full-length human GR as bait, which is not possible in conventional yeast two-hybrid approaches. Secondly, it allowed the somewhat unusual step of anchoring the GR in the membrane, keeping it cytoplasmic even in the presence of hormone. Our intention in doing so was to facilitate interactions with cytoplasmic proteins. While most of the identified interaction candidates were indeed cytoplasmic, a number of nuclear proteins were identified as well, raising the question how these proteins were able to make contact with the membrane-bound receptor.

Figure 8 ZKSCAN4 dampens glucocorticoid responsiveness of a chromosomally integrated MMTV-promoter. (A) Dex induction of a chromosomally integrated MMTV-VNP reporter. Jurkat-GR and Jurkat-GR MMTV-VNP cells were cultured for 14 h in the presence or absence of dex as indicated, and fluorescence intensity was measured by flow cytometry. (B) Effect of ZKSCAN4 on dex induction of the MMTV-VNP reporter. Jurkat-GR MMTV-VNP cells and Jurkat-GR MMTV-VNP ZKSCAN4 cells were cultured for 14 h in the presence of dex and fluorescence intensity was measured by flow cytometry. Both graphs represent overlays of curves showing relative fluorescence intensity against relative number of cells.

The simplest explanation would be that overexpression in the yeast cell resulted in a certain amount of the protein being cytoplasmic. We expected the presence of a glucocorticoid to be essential during the screening procedure. This proved not to be the case. Although much of the screening was done in the presence of deoxycorticosterone, which, in contrast to dex, was reported to be able to pass the yeast cell wall (Garabedian & Yamamoto 1992), we did not identify any candidate demonstrating strictly ligand-dependent interaction. Positive clones remained positive, even in the absence of deoxycorticosterone. This was also true for nuclear proteins. Why did not more of the many known GR-interactors turn up in our screen? One limitation is probably the nature of our cDNA library, which was oligo-dT primed. The farther away the encoded interaction domain is from the poly-A tail, the less its chance to be represented in the library. Small interacting proteins or protein parts containing C-terminal interaction domains are far more likely to be identified than larger proteins with N-terminal

interaction domains. In addition, abundantly expressed yeast proteins may be sometimes crowding out competing similar human candidate proteins.

Of the proteins listed in Table 1, several are quite hydrophobic and belong to classes of proteins that have been found repeatedly in conventional yeast two-hybrid screens, e.g., ribosomal proteins and elongation factors. Although this does not exclude the possibility that they are GR interactors, a majority of them is likely to have been isolated due to unspecific interactions.

Of all assessed candidate proteins, interaction with the GR in human cell lines was most consistently confirmed for ZKSCAN4. As interaction was found in both yeast and human cell lines, it is likely to be direct, although it remains possible that interaction occurs via a third protein or a protein complex, as long as an analogous protein or complex is present in human as well as in yeast cells. According to the NCBI-database of expressed sequence tags (ESTs, June 2008), ZKSCAN4 is moderately expressed in many tissues, including, in descending order, cervix (82 transcripts per million),

blood (64), tonsils (58), testis (51) and trachea (38), kidney (37), adrenal gland (30), mouth (29), skin (28), lung, (23), brain (19), spleen (18), uterus (17) and absent, e.g., in liver, intestine, and muscle.

While ZKSCAN4 was isolated as an interaction partner of the GR, it conceivably might also interact with other members of the nuclear receptor superfamily. To address this question, we specifically tested the PR, which, in contrast to GR, was not co-precipitated with ZKSCAN4. Obviously, this does not prove specificity for the GR, and further members of the superfamily will have to be tested individually.

ZKSCAN4 is a member of a large group of human zinc finger proteins that contain a KRAB- and a SCAN-box. Some of them are thought to bind to DNA via their zinc finger domain, followed by transcriptional repression via the KRAB-box. The SCAN-box is thought to mediate homo- and hetero-dimerization with other SCAN-containing proteins (Williams *et al.* 1999). While most of these proteins have not been functionally characterized so far, individual members of this group have been reported to have roles in binding and splicing of RNA (Grondin *et al.* 1996), or to repress transcription from RNA polymerase I-, II- and III-promoters (reviewed by Urrutia 2003).

While co-localization of ZKSCAN4 with Y12, which decorates regions enriched in splicing factors would have suggested a role in splicing, no such association was found. The only potential, and very vague, hint on function from our co-localization experiments was an overlap in localization of ZKSCAN4 with nuclear regions showing histone H3 mono-methylated on lysine 4, which might suggest an involvement in chromatin regulation (Sims *et al.* 2003, Schneider *et al.* 2004).

To assess an influence of ZKSCAN4 on the induction of the MMTV-promoter, which contains two glucocorticoid response elements, we selected two approaches. In a classical transient transfection assay with a naked-DNA reporter plasmid, we did not find any direct effect of ZKSCAN4 on glucocorticoid induction. However, if a potential repressive effect of ZKSCAN4 were to rely on a chromatin-condensing function, this cannot be detected by the transient transfection of an episomal reporter plasmid, but requires integration of the MMTV-reporter construct into a chromosome (Deroo & Archer 2001). We therefore modified a GR-overexpressing Jurkat subline (Helmberg *et al.* 1995), adding a chromosomally integrated MMTV-driven VNP-reporter. In this setting, VNP fluorescence indicating MMTV-promoter activity was markedly reduced in the cells overexpressing ZKSCAN4. The difference in ZKSCAN4's ability to affect the identical promoter, dependent on the promoter's naked versus chromatin-embedded state, argues for a chromatin-dependent repressive effect of ZKSCAN4. Whether this effect is

specifically directed to glucocorticoid responsive genes or of a more general nature remains to be investigated.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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