A novel partner for D-type cyclins: protein kinase A-anchoring protein AKAP95

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Using a yeast interaction screen to search for proteins that interact with cyclin D3 in thyroid gland, we identified the cAMP-dependent AKAP95 (protein kinase A-anchoring protein 95). AKAP95 is a scaffolding protein that primarily co-fractionates with the nuclear matrix, whereas a minor fraction associates with chromatin in interphase cells. In co-transfected Chinese-hamster ovary cells, AKAP95 strongly interacted with the three D-type cyclins, but not with CDK4 (cyclin-dependent kinase 4) or with p27^{kip1}. CDK4 displaced the interaction between cyclin D3 and AKAP95, suggesting that AKAP95 could not be the elusive bridging adaptor between D-type cyclins and CDK4 or play a role in the regulation of cyclin D3–CDK4 activity. Interaction between endogenous AKAP95 and cyclin D3 or cyclin D1 was detected in

canine thyrocytes, human fibroblasts and NIH-3T3 cells. As both AKAP95 and cyclins D were recently reported to associate with minichromosome maintenance proteins [Eide, Tasken, Carlson, Williams, Jahnsen, Tasken and Collas (2003) J. Biol. Chem. 278, 26750–26756; Gladden and Diehl (2003) J. Biol. Chem. 278, 9754–9760], we hypothesize that the interaction between AKAP95 and D-type cyclins might serve to facilitate the emerging regulatory role of cyclin D–CDK4 in the formation of the pre-replication complex at the DNA replication origins.

Key words: cyclin-dependent kinase 4 (CDK4), cyclin D, pre-replication complex, protein kinase A-anchoring protein 95 (AKAP95), two-hybrid system.

INTRODUCTION

Cell-cycle progression in upper eukaryotes requires sequential and ordered formation, activation and then inactivation of different complexes of CDKs (cyclin-dependent kinases). D-type cyclin–CDK4/CDK6 complexes phosphorylate and inactivate growth/tumour suppressor proteins of the pRb (retinoblastoma susceptibility protein) family (p105^{Rb}, p107 and p130^{Rb2}) [1–6] to initiate the passage through the ‘restriction point’ in mid-to-late G₁ phase beyond which the cell cycle is committed to proceed autonomously [7–9]. It was initially considered that mitogens activate CDK4/CDK6 by inducing at least one D-type cyclin (D1–D3, which are differentially expressed in different tissues) [7]. However, D-type cyclins and CDK4 assemble poorly *in vitro* [10,11] or in the absence of mitogenic stimulation [12,13]. Moreover, D-type cyclins and CDK4 do not contain an NLS (nuclear localization signal), whereas the activation of CDK4 requires phosphorylation on Thr¹⁷² by the CDK-activating kinase, cyclin H–CDK7 [14], which is nuclear, similar to the CDK4 substrates [15]. Although there is still a consensus to consider CDK ‘inhibitors’ of the CIP/KIP (CDK-interacting protein/kinase-inhibitory protein) family as inhibitors of CDK2 [16], p27^{kip1} and p21^{cip1}, which possess an NLS and distinct binding domains for cyclins and CDKs, have been found to be associated with a pRb-kinase activity [17,18]. They can stabilize cyclin D–CDK4 complexes *in vitro* or in co-transfected cells [11] and target these complexes to the nucleus [11,19]. p21^{cip1} and p27^{kip1} were even shown to be essential for these stimulatory functions [20]. However, other authors have recently concluded that these proteins are not absolutely required for the mitogen-dependent assembly of cyclin D3–CDK4 [21] and cyclin D1–CDK4 [22], and that only the

minor fraction of cyclin D3–CDK4 complexes devoid of CIP/KIP proteins are active as pRb-kinases [21].

The crucial importance of the regulated assembly and nuclear import of D-type cyclin–CDK4 complexes has been exemplified by the delineation of cell-cycle regulatory mechanisms in the experimental system of dog thyroid epithelial cells. In this physiologically relevant model [23], the cAMP-dependent mitogenic stimulation elicited by thyrotropin (TSH; thyroid-stimulating hormone) does not up-regulate D-type cyclins, but it specifically requires the high expression of cyclin D3 [13], itself supported by the co-mitogen insulin/insulin-like growth factor-1 [24]. cAMP promotes the assembly, nuclear translocation and then activation (through CDK4 phosphorylation) of cyclin D3–CDK4 complexes [13,25]. Moreover, TSH and cAMP enhance the expression of nuclear p27^{kip1} [26], which associates with cyclin D3–CDK4 complexes [27] and supports the phosphorylation and pRb-kinase activity of cyclin D3-bound CDK4 [28]. Nevertheless, p27^{kip1} does not appear to be instrumental in the cAMP-dependent formation of cyclin D3–CDK4 complexes, because the moderate increase in p27^{kip1} induced by cAMP appears insufficient to explain the 10–20-fold increase in cyclin D3–CDK4 complexes, and because transforming growth factor β inhibits the activity of cyclin D3–CDK4 by preventing its nuclear import and its binding to p27^{kip1} without affecting the assembly of this complex [27,28]. No modifications of phosphorylation of cyclin D3 or p27^{kip1} were detected during the cAMP-dependent assembly and activation of the cyclin D3–CDK4–p27^{kip1} holoenzyme [25,28].

This has raised the possibility of the involvement of other cyclin D3-bound proteins in the elusive mechanisms of the cAMP-dependent formation and subsequent activation of the cyclin D3–CDK4 complex. To investigate this issue, a two-hybrid screening

Abbreviations used: PKA, protein kinase A; AKAP95, PKA-anchoring protein 95; CDK, cyclin-dependent kinase; CHO, Chinese-hamster ovary; CIP/KIP, CDK-interacting protein/kinase-inhibitory protein; DMEM, Dulbecco's modified Eagle's medium; FCS, foetal calf serum; HA, haemagglutinin; MCM, minichromosome maintenance; NLS, nuclear localization signal; pRb, retinoblastoma susceptibility protein; preRC, prereplication complex; TSH, thyroid-stimulating hormone (thyrotropin).

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The nucleotide sequence of dog AKAP95 has been submitted to the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY382602.

from a dog thyroid cDNA library was performed using the full-length human cyclin D3 as a bait, and AKAP95 [protein kinase A (PKA)-anchoring protein 95] was identified as a new cyclin D-associated protein.

EXPERIMENTAL

Plasmids and antibodies

The dog AKAP95 coding sequence was amplified by PCR and cloned into yeast two-hybrid vector pPC86 (gift from P. Chevray, University of Texas, Houston, TX, U.S.A. and D. Nathans, Howard Hughes Medical Institute, Baltimore, MD, U.S.A.) and pcDNA3.HIS (Invitrogen, Merelbeke, Belgium). Similarly, we cloned full-length cyclin D3 cDNA in the yeast two-hybrid vector pPC97 (gift from P. Chevray and D. Nathans) and the pcDNA3.HA vector (where HA stands for haemagglutinin). The full-length p27^{kip1} was cloned into pcDNA3 vector (Invitrogen). pXD1, pXD2 and PCMV.HA-CDK4 were kindly provided by J. Bartek (Institute of Cancer Biology, Copenhagen, Denmark).

The following antibodies were used: a mouse monoclonal antibody against HA (Roche Diagnostics, Mannheim, Germany), a mouse monoclonal antibody against His (ClonTech, Palo Alto, CA, U.S.A.), a rabbit (R-146) polyclonal antibody against AKAP95 and rabbit polyclonal antibodies against CDK4 and p27^{kip1} (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), a monoclonal antibody against human AKAP95 (Transduction Laboratories, Palo Alto, CA, U.S.A.), and monoclonal antibodies against human cyclin D3 (DCS-22 and DCS-28), cyclin D1 (DCS-6) and cyclin D2 (DCS-5) [29]. Horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies from goat (both from Amersham Biosciences, Little Chalfont, Bucks., U.K.) were used as secondary reagents to detect monoclonal or polyclonal antibodies respectively.

Two-hybrid screenings and constructs

The complete sequence of human cyclin D3 cDNA was cloned by PCR in-phase downstream of the Gal4 DNA-binding domain in the yeast two-hybrid vector pPC97. The construction was verified by DNA sequencing. The cDNA library synthesized from dog thyroid poly(A)⁺ (polyadenylated) RNAs (Superscript plasmid system; Gibco Invitrogen Corporation, Merelbeke, Belgium) was fused to the Gal4 transcription-activating domain in the yeast two-hybrid vector pPC86 [30]. The yeast host strain used for the screening and the reconstruction steps was PJ69-4A (*MAT a, ade 2 trp 1-Δ901 leu 2-3,112 ura 3-52 his 3-200 gal-4Δ gal-80Δ LYS2::GAL1-HIS3 ADE2::GAL2-ADE2 met1::GAL7-LACZ*). For the screening, PJ69-4A harbouring pPC97-cyclin D3 was transformed with the dog thyroid cDNA library in pPC86. Transformants were first selected on αHIS medium, then on αADE and finally reconstructed for specificity.

Cell cultures, transfections and immunoprecipitation of protein complexes

Dog thyrocytes, seeded as follicles (2×10^4 cells/cm²), were cultured in monolayer in the following medium [31]: DMEM (Dulbecco's modified Eagle's medium) + Ham's F12 medium + MCDB104 medium (2:1:1, by vol.; Life Technologies Laboratories, Paisley, Renfrewshire, Scotland, U.K.), supplemented with 40 µg/ml ascorbic acid, 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Fungizone). The medium was changed every other day. On day 4, the quiescent cells were treated with 1 m-unit/ml bovine TSH (Sigma, St. Louis, MO, U.S.A.) and 5 µg/ml insulin (Sigma). Human diploid fibroblasts

(IMR-90; A.T.C.C., Manassas, VA, U.S.A.) were obtained at population doubling 24 and grown for not more than 40 total population doublings in DMEM (Gibco), supplemented with antibiotics and 10% (v/v) FCS (foetal calf serum). After reaching semi-confluency, they were synchronized by starvation in 0.2% FCS for 3 days. Quiescent IMR-90 cells were then growth-stimulated by the addition of 20% FCS. NIH-3T3 cells were maintained in DMEM supplemented with antibiotics and 10% FCS. CHO (Chinese-hamster ovary) cells were transfected using Fugene 6 (Roche Diagnostics) with full-length His-tagged dog AKAP95 and full-length human HA-tagged cyclin D3, human HA-tagged CDK4, human cyclin D1, human cyclin D2 or dog p27^{kip1}. Cells were harvested 48 h after transfection.

Subconfluent cultures, in 100 mm Petri dishes that contained the same number of cells, were washed 20 h after stimulation with calcium- and magnesium-free PBS and lysed in 1 ml of Nonidet P40 lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.5% Nonidet P40, 50 mM NaF, 1 mM sodium orthovanadate, dithiothreitol and protease inhibitors. The homogenized (glass/glass) cellular lysate was sonicated twice, precleared with Protein G-Sepharose (Amersham Biosciences, Uppsala, Sweden) and then incubated at 4 °C for 3 h with 50 µl of Protein G-Sepharose, which had been preincubated overnight with 4 µg of either DCS-28 (cyclin D3), DCS-11 (cyclin D1) or AKAP95 R-146 rabbit polyclonal antibody. After three washes, the immune complexes were suspended in SDS lysis buffer, boiled for 10 min and analysed by SDS/PAGE (8.5% gel). The proteins were immunodetected by Western blotting as described in the Figure legends.

RESULTS

Identification of AKAP95 as a cyclin D3-interacting protein

To identify proteins that interact with cyclin D3, we used the yeast two-hybrid system with human cyclin D3 cloned in the pPC97 vector as a bait. A dog thyroid cDNA library cloned in the pPC86 vector was screened as described in the Experimental section. Among 1.5×10^6 transformants, 63 colonies were His⁺ Ade⁺. To eliminate the false positives, library plasmids were re-introduced into wild-type yeasts or in yeasts expressing GAL4DBD (DNA-binding domain) fused to an unrelated protein or alone (Figure 1A). Among the first 63 His⁺ Ade⁺ colonies, 19 showed specificity for the bait. One of the positive 1.3 kb cDNA fragment encoded the N-terminal (1–458 amino acids) region of AKAP95 (Figure 1B).

Isolation and sequence of dog AKAP95

We cloned the full-length dog cDNA encoding AKAP95, a 698-amino-acid protein, by PCR from a dog thyroid cDNA library. As shown in Figure 2, the dog AKAP95 (accession no. AY382602) presents 81% identity with the human AKAP95 protein (accession no. NP_005848.1). The dog protein is longer by six amino acids compared with the human counterpart owing to various insertions. The dog AKAP95 protein contains (Figure 2) two Zn fingers (framed), an NLS (underlined) and an RII-binding domain (bold). The proposed RII-binding domain is conserved between dog and human, with the exception of one amino acid change from Asp to Glu at position 5 of a putative amphipathic helix, which can be predicted.

AKAP95–cyclin D3 interaction in intact cells

Co-transfections of cyclin D3 and AKAP95 in CHO cells were performed to confirm the interaction between the two proteins. The His-tagged full-length dog AKAP95 protein was

Figure 1 AKAP95 binds to cyclin D3 in two-hybrid experiments

(A) Yeast expressing GAL4DBD (DNA-binding domain), GAL4DBD-unrelated protein or GAL4DBD-cyclin D3 were transformed with GAL4AD (activation domain)-AKAP95 and plated on the medium lacking tryptophan, leucine and adenine or histidine. (B) Schematic representation of AKAP95 with its functional and binding domains. Numbers refer to amino acid positions. NMTS, nuclear matrix targeting sequence; biNLS, bipartite NLS; ZF, Zn finger; PKA, PKA-binding domain.

co-expressed in CHO cells with HA-tagged full-length human cyclin D3. Cells were lysed 48 h after transfection. The expression of AKAP95 and cyclin D3 in whole-cell extracts was verified (Figure 3) in untransfected cells (lane 1), in cells transfected with His-AKAP95 and HA-empty vector (lane 2), HA-cyclin D3 and His-empty vector (lane 3) and both His-AKAP95 and HA-cyclin D3 (lane 4). Cyclin D3 was then isolated by immunoprecipitation using monoclonal anti-(cyclin D3) antibody DCS-28. Immunoprecipitated cyclin D3 was detected by Western blotting with the anti-cyclin D3 antibody DCS-22. Using anti-AKAP95 immunoblot analysis, we showed (Figure 3) that AKAP95 co-immunoprecipitated with cyclin D3 only from cells transfected with both cyclin D3 and AKAP95.

AKAP95 interacts with all the D-type cyclins

To confirm the previous results and to investigate the possibility of AKAP95 forming complexes with other cyclins D, or with their known partners CDK4 or p27^{kip1}, the immunoprecipitation of AKAP95 was realized in CHO cells transfected with HA-tagged full-length human cyclin D3 (Figure 4A), full-length human cyclin D1 (Figure 4B), full-length human cyclin D2 (Figure 4C), HA-tagged full-length human CDK4 (Figure 4D) or full-length dog p27^{kip1} (Figure 4E). Cells were lysed 48 h after transfection. AKAP95 was immunoprecipitated with anti-AKAP95 rabbit polyclonal antibody and the precipitates were examined for the presence of cyclin D3 (Figure 4A), cyclin D1 (Figure 4B), cyclin D2 (Figure 4C), CDK4 (Figure 4D) or p27^{kip1} (Figure 4E) using the corresponding antibodies. These results confirmed the interaction

Dog	1	MEQYGGYGAWAGPTNTQAGYGTGVASWQGYENNYNYGAQNTSVTTGASYSYGPASWET	60
Human	1	M+QYGGYGAWAGPTNTQAGYGTGVASWQGYENNYNYGAQNTSVTTGA+YSYGPASWE	60
Dog	61	TKASDS-LGAGGPAVHMASYGPEPCTDSDSLIAKINQRLDMMSKEGGGSGGGEGMQ	119
Human	61	KA+D L AG PA+HMASYGPEPCTDSDSLIAKINQRLDMMSKEGGGSGGGEGIQ	120
Dog	120	DRESSFRFQSYESYDSRCLPEHHPYRPSYSYDYDFDMPDRNGGFSQYDCROPARER	179
Human	121	DRESSFRFQ+ESYDSR CLPEH+PYRPSYSYDY+FD DRNG F QY +CRDPARER	180
Dog	180	GSLDGFMRGRGQGGQGRFQDRSNPSTFMRSDFF+PP+ASSEPLSAPWPELNYVGRGLG	239
Human	181	GSLDGFMRGRGQGG---RFQDRSNPSTFMRSDFF+PP+ASSEPLSPWPELNYVGRGLG	236
Dog	240	GPSRPPPSLFSQSMAPDFGVGMQAGGYDYNVPGYCGRSQTRMRDR---PRRRGDFRC	297
Human	237	GPSRPPPSLFSQSMAPD+GVGMQAGGYD++PYGCGRSQ RMRDR P+RRGDFR	296
Dog	298	GPDGGRKRKQIYDEYDAKLARADSEGDSENDGADFRSGDEEFGEDEFFDGRQ	357
Human	297	GPDG GRKRKQ Q+Y+EPD KLAR DSEGDSEND D A DFRSGDEEF+GEDE D GRQ	356
Dog	358	RGEKDEDEDEVKKRREKQRDRMRDRADRIQFACSVCKFRSFEDEIQKHLQSKFHK	417
Human	357	RGEK+DED+VKKRREKQRDRMRDRADRIQFACSVCKFRSF+DEIQKHLQSKFHK	416
Dog	418	TLRFISTKLDPKTVFLQYIVNRRKKIEKRQELMEKESTKPKDPFKIGQEHFFPKI	477
Human	417	TLRFISTKLDPKTVFLQYIVNRRKKIEKRQELMEKE+ KPKDPFKIGQEHFFPKI	476
Dog	478	EAHCLACDMLIPAQQLLQRLHLSVDHNNRRLAAEQFKKTSLVHVARSVLNNRHVKML	537
Human	477	EAHCLACDMLIPAQ QLLQRLHLSVDHNNRRLAAEQFKKTSLVHVA+SVLNNRHVKML	536
Dog	538	EKYLKGEDPFTSEAVDPEIEGDDTLGGE-KEETPEEVAEVLAEVITAARAVDGE	596
Human	537	EKYLKGEDPFTSE VDPPE+EGDD LGGE K+ETPEEVA+VLAEVITAARAVDGE	596
Dog	597	PEGSPTPEGQGPVDTAEATHSPHSEQLVEVETPCGAAPKGDANAEAGGETTEAAGEEQ	656
Human	597	PE S P E +GP DTAEA P +EQL+E + PCG A EKG A + E EA +	654
Dog	657	EVVAKTDGAVDGTVRGAAPPVVTEKAEQTEAEKPDVTPE	698
		+ A + A APAP + + EQT+AE KD PTE	
Human	655	TMAAEESA-----QTRVAPAPAAADAEVETDAESKDAVFTE	692

Figure 2 Sequence comparison between dog and human AKAP95

The percentage identity is 81% between the two proteins. Numbers indicate the respective position in the amino acid sequence. The biNLS (underlined), two putative Zn fingers (framed) and the RII-binding domain (bold) are indicated.

CHO cells	1	2	3	4
HA-D3	-	-	+	+
HIS-AKAP	-	+	-	+

Figure 3 AKAP95 and cyclin D3 associate in CHO cells

CHO cells were untransfected (lane 1), co-transfected with His-tagged dog AKAP95 and HA-empty vector (lane 2), HA-cyclin D3 and His-empty vector (lane 3) or His-AKAP95 and HA-cyclin D3 (lane 4). Cells were harvested and WCE (whole-cell extracts) were resolved by SDS/PAGE and immunoblotted with anti-His and anti-HA. The extracts were then IP (immunoprecipitated) with DCS-28 (anti-cyclin D3 antibody) and the resulting proteins were immunoblotted (IB) with anti-AKAP95 and anti-cyclin D3 antibodies (IP: α-D3).

Figure 4 AKAP95 associates with all D-type cyclins in CHO cells, but not with CDK4 and p27^{kip1}

The immunoprecipitation (IP) of AKAP95 was realized with rabbit polyclonal antibody (Santa Cruz Biotechnology) in CHO cells transfected with His-AKAP95, and either with HA-tagged full-length human cyclin D3 (**A**), full-length human cyclin D1 (**B**), full-length human cyclin D2 (**C**), HA-tagged full-length human CDK4 (**D**) and full-length dog p27^{kip1} (**E**). Cells were lysed 48 h after transfection and the precipitates were examined by immunoblotting (IB) for the presence of cyclin D3 (**A**), cyclin D1 (**B**), cyclin D2 (**C**), CDK4 (**D**) and p27^{kip1} (**E**). WCE, whole-cell extract.

between AKAP95 and cyclin D3 and showed that AKAP95 can interact with all the D-type cyclins, but not with CDK4 or p27^{kip1}.

CDK4 recruits cyclin D3 from the AKAP95–cyclin D3 complex

To test whether the interaction between cyclin D3 and AKAP95 can influence the formation of cyclin D3–CDK4 complexes, the immunoprecipitation of cyclin D3 was performed in CHO cells triple-transfected with HA–cyclin D3, His-AKAP95 and HA-CDK4 (Figure 5). Cells were lysed 48 h after transfection and proteins were detected by Western blotting using the corresponding antibodies (Figure 5). Immunoprecipitation of cyclin D3 was performed with DCS-28 antibody and the precipitate was examined for the presence of AKAP95 and CDK4. Using anti-AKAP95 immunoblot analysis, we observed that the interaction of AKAP95 with cyclin D3 was impaired when

transfected CDK4 was present (lane 8). In contrast, the interaction of CDK4 with cyclin D3 was not affected by high AKAP95 expression. This result suggests that CDK4 competes with AKAP95 in binding to cyclin D3 and that CDK4 can recruit cyclin D3 from AKAP95–cyclin D3 complexes.

AKAP95 interacts with D-type cyclins in intact cells

To demonstrate the interaction between cyclin D3 and AKAP95 in a physiological context with endogenously expressed proteins, the immunoprecipitation experiments were first performed in primary cultures of dog thyroid cells. In dog thyrocytes, stimulated to proliferate by TSH in the presence of insulin, cyclin D3 is the main D-type cyclin involved in CDK4 activation and DNA synthesis induction [13]. As shown previously [24], TSH alone paradoxically repressed cyclin D3 accumulation. This inhibition

Figure 5 CDK4 recruits cyclin D3 from AKAP95–cyclin D3 complexes

CHO cells were untransfected (lane 1) or co-transfected with His-tagged dog AKAP95 and HA-empty vector (lane 2), HA–cyclin D3 and HIS–empty vector (lane 3), HA–AKAP95 and HA–cyclin D3 (lane 5), His–AKAP95 and HA–CDK4 (lane 6), HA–cyclin D3 and HA–CDK4 (lane 7) and His–AKAP95, HA–cyclin D3 and HA–CDK4 (lane 8). Cells were harvested and WCE (whole-cell extracts) were resolved by SDS/PAGE and immunoblotted with anti-AKAP95, anti-cyclin D3 or anti-CDK4 antibody (WCE). The extracts were then immunoprecipitated (IP) with DCS-28 (anti-cyclin D3 antibody) and the resulting proteins immunoblotted (IB) with anti-AKAP95, anti-CDK4 and anti-cyclin D3 antibodies (IP: α -D3).

was in part overridden by insulin, which stimulates cyclin D3 mRNA and protein accumulation and supports the formation by TSH of cyclin D3–CDK4 complexes required for DNA synthesis. AKAP95 was faintly detected in whole-cell extracts of dog thyrocytes. There was no detectable influence of insulin or TSH on its expression (Figure 6A). Cyclin D3 was immunoprecipitated with the monoclonal antibody DCS-28 and the precipitates were examined for the presence of AKAP95. In the experiment shown, AKAP95 was detected in cyclin D3 immunoprecipitates from unstimulated dog thyrocytes and from cells stimulated with both insulin and TSH (lane 4), but not from cells that abundantly express cyclin D3 in response to insulin alone. The interaction between AKAP95 and cyclin D3 was confirmed in two other primary cultures of dog thyrocytes, but the modulations were not consistently observed. Attempts to immunoprecipitate AKAP95 from thyroid cells failed, probably due to weak expression of AKAP95 in dog thyroid.

The interaction between endogenously expressed AKAP95 and cyclin D1 was examined in IMR-90 human fibroblasts and NIH-3T3 cells. IMR-90 cells were stimulated with 20 % FCS for 16 h and whole-cell extracts were examined for the presence of endogenous cyclin D1 and AKAP95 (Figure 6B). Cyclin D1 was expressed in quiescent (control, C) cells and further accumulated in cells stimulated with 20 % FCS for 16 h (stimulated, S), when a maximum number of cells were in late G₁ phase. AKAP95 was weakly expressed in IMR-90 cells and its expression did not seem to be modulated on stimulation. AKAP95 was detected in cyclin D1 immunoprecipitates from either control (C) or stimulated cells (S) (Figure 6B). In contrast, cyclin D1 was very faintly detected in AKAP95 immunoprecipitates from serum-stimulated IMR-90 cells (results not shown).

Both in thyrocytes and in IMR-90 cells, the low amount of immunoprecipitated AKAP95 could be explained by the low expression of AKAP95 protein in these cells. Other authors have also mentioned that the detection and immunoprecipitation of endogenous AKAP95 requires large amounts of cells [32]. Higher apparent amounts of AKAP95 protein were

Figure 6 AKAP95 binds D-type cyclins in intact cells

(A) Thyroid cells in primary culture were maintained for 4 days in control medium without insulin before stimulation for 20 h by different agents. Immunoprecipitation (IP) of cyclin D3 was realized with the mouse monoclonal antibody DCS-28, proteins were detected by Western blotting using a monoclonal anti-AKAP95 antibody and the DCS-22 monoclonal antibody against cyclin D3. Lanes: α , control without insulin; T, TSH (1 m-unit/ml); I, insulin (5 μ g/ml); IT, insulin (5 μ g/ml) + TSH (1 m-unit/ml). (B) Human fibroblast IMR-90 cells were maintained in a quiescent state (C) or stimulated with 20 % FBS for 16 h (S). Cells were harvested and WCE (whole-cell extracts) were resolved by SDS/PAGE and immunoblotted (IB) with anti-AKAP95 and anti-cyclin D1 antibodies. Immunoprecipitation of cyclin D1 was realized with monoclonal DCS-11 antibody and the (co)immunoprecipitated proteins were IB with anti-AKAP95 and anti-cyclin D1 antibodies. (C) NIH-3T3 cells were maintained in a medium supplemented with 10 % FCS. Cells were harvested and WCE were resolved by SDS/PAGE and IB with anti-AKAP95 and anti-cyclin D1 antibodies. The extracts were subjected to immunoprecipitation with R-146 (anti-AKAP95 antibody) and the (co)immunoprecipitated proteins were IB with anti-AKAP95 and anti-cyclin D1 antibodies.

observed in NIH-3T3 cells (Figure 6C). Consistent with this, co-immunoprecipitation of endogenous AKAP95 and cyclin D1 was clearly detected in these cells (Figure 6C), confirming the presence of cyclin D1–AKAP95 complexes in intact cells.

DISCUSSION

To identify proteins that interact with cyclin D3 and influence the formation, translocation and/or activation of the cyclin D3–CDK4 complex, as observed in thyroid cells after insulin and TSH (cAMP) co-stimulation, we performed a yeast two-hybrid screen of a dog thyroid cDNA library using the full-length human cyclin D3 as a bait. Among positive cDNAs obtained by this approach, we unexpectedly identified AKAP95 as a cyclin D3-binding protein. Domain(s) responsible for this interaction are located in the N-terminal part of AKAP95 (amino acids 1–458) and thus do not overlap the PKA-binding domain. After cloning of the full-length dog AKAP95, we confirmed this interaction by co-expression in CHO cells. Furthermore, we were able to co-immunoprecipitate endogenous AKAP95 and cyclin D3 from some primary cultures of dog thyroid cells, a system characterized by a specific requirement for high cyclin D3 expression in the cAMP-dependent triggering of DNA replication of these cells. We next observed that the three D-type cyclins can associate with AKAP95, suggesting a wider importance of this interaction for cell-cycle progression, and we confirmed the interaction between endogenously expressed cyclin D1 and AKAP95 in human IMR-90 fibroblasts and NIH-3T3 cells.

AKAP95 is a 95 kDa AKAP, which is reported to be exclusively nuclear during interphase [33,34]. AKAP95 co-fractionates primarily with the nuclear matrix in interphase, whereas a minor fraction associates with chromatin [32,35]. At mitosis, AKAP95 mostly co-fractionates with chromosomes and associates with RII α to form a complex necessary to maintain chromosomes in a condensed form [32]. As the binding of PKA to AKAP95 is strictly restricted to the mitosis phase [32], depending on RII α phosphorylation by CDK1 [36], we excluded the hypothesis that it might specifically intervene in the elusive mechanisms mediating the successive actions of cAMP on the cyclin D3–CDK4 complexes in thyroid cells [13,25]. It is also unlikely that AKAP95 would have functioned as the proposed bridging adaptor between cyclin D and CDK4, because it did not bind to CDK4 and its interaction with cyclin D3 was even completely displaced by over-expressed CDK4 in co-transfected CHO cells. For the same reasons, AKAP95 was unlikely to play any role in the regulated activation of the cyclin D3–CDK4 complex through its phosphorylation by CDK activating kinase. Further investigations are necessary to map the exact regions of interaction between cyclin D3 and AKAP95 and to determine whether AKAP95 and CDK4 compete for the same interaction domain on cyclin D3.

As a nuclear matrix scaffolding protein, AKAP95 associates with p68 RNA helicase, which has been suggested to play a role in the chromatin remodelling reaction, indicating the possibility that AKAP95 could be involved in the assembly of hormone-responsive transcriptional complexes [35]. Recently, an associate of Myc-1, a binding protein and possible co-activator for the c-Myc transcriptional factor, has been found to physically interact with AKAP95 [37]. As D-type cyclins, independent of CDK4 activation and pRb phosphorylation, have been described as cofactors for oestrogen and androgen nuclear receptors (cyclin D1) [38–41] or retinoic acid receptors (cyclin D3) [42], the involvement of the interaction of D-type cyclins with AKAP95 in some transcriptional functions of D-cyclins cannot be excluded.

During the course of this study, it has been described that AKAP95 also directly interacts with MCM2 (minichromosome

maintenance protein 2), a component of the hexameric MCM2–MCM7 DNA helicase preRC (prereplication complex), possibly providing a scaffold for MCM2 on the chromatin [43]. Moreover, another recent study has demonstrated the incorporation of catalytically active D-type cyclin–CDK4 into chromatin-bound MCM7 complexes during G₁ phase progression, apparently to phosphorylate pRb and dissociate inhibitory pRb–MCM7 complexes, thereby facilitating the establishment of the preRC [44]. Collectively, these studies lead us to consider the possibility that the presently demonstrated interaction between AKAP95 and D-type cyclins might serve to facilitate this regulatory function of cyclin D–CDK4 on the formation of the preRC at the DNA replication origins. The formation of the cyclin D–CDK4 complex appeared to displace the cyclin D–AKAP95 interaction (Figure 5). Therefore, on mitogenic stimulation, AKAP95-anchored cyclin D might be mobilized in the close vicinity of chromatin-bound preRC by its assembly into active cyclin D–CDK4 complexes, which then could immediately associate with MCM7 [44] and thus contribute to regulate directly the initiation of DNA replication, e.g. by triggering the dissociation of pRb–MCM7 complexes [44,45]. The exact role of cyclins D and AKAP95 in preRC remains to be determined. The fact that MCM7 is not a substrate for cyclin D1–CDK4 [44] does not exclude the possibility that other cyclin D–CDK4 substrates exist within the preRC. These points need to be investigated further to elucidate the nature and function of D-type cyclin–AKAP95 interactions.

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