Distinct but overlapping domains of AKAP95 are implicated in chromosome condensation and condensin targeting

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A-kinase (or PKA)-anchoring protein AKAP95 is a zinc-finger protein implicated in mitotic chromosome condensation by acting as a targeting molecule for the condensin complex. We have identified determinants of chromatin-binding, condensin-targeting and chromosome-condensation activities of AKAP95. Binding of AKAP95 to chromatin is conferred by residues 387–450 and requires zinc finger ZF1. Residues 525–569 are essential for condensation of AKAP95-free chromatin and condensin recruitment to chromosomes. Mutation of either zinc finger of AKAP95 abolishes condensation. However, ZF1 is dispensable for condensin targeting, whereas the C-terminal ZF2 is required. AKAP95 interacts with *Xenopus* XCAP-H condensin subunit *in vitro* and *in vivo* but not with the human hCAP-D2 subunit. The data illustrate the involvement of overlapping, but distinct, domains of AKAP95 for condensin recruitment and chromosome condensation and argue for a key role of ZF1 in chromosome condensation and ZF2 in condensin targeting. Moreover, condensin recruitment to chromatin is not sufficient to promote condensation.

INTRODUCTION

Condensation of chromosomes at mitosis requires the highly conserved 13S condensin complex (Hirano, 2000). In humans, the condensin complex consists of two structural maintenance of chromosomes (SMC) subunits (hCAP-C and hCAP-E) and three regulatory non-SMC subunits (hCAP-D2/CNAP1, hCAP-G and hCAP-H) (Schmiesing *et al.*, 1998, 2000; Kimura *et al.*,

2001). The 13S condensin complex isolated from *Xenopus* egg extracts displays ATPase activity *in vitro*, which introduces positive supercoils into DNA and thereby probably assists in chromosome condensation (Kimura and Hirano, 1997; Kimura *et al.*, 1999). Condensin recruitment to chromatin at mitosis correlates with mitotic phosphorylation of the non-SMC subunits (Hirano *et al.*, 1997). Additional components regulating anchoring of condensins to chromosomes are also probably implicated (Kimura *et al.*, 1998; Schmiesing *et al.*, 2000).

Another important factor for mitotic chromosome condensation is the cAMP-dependent protein kinase (PKA or A-kinase) anchoring protein AKAP95. AKAP95 is a 95 kDa nuclear protein of the nuclear matrix, harboring two zinc fingers of unknown function (designated ZF1 and ZF2) in its C-terminal half, upstream of the PKA-binding domain (Eide *et al.*, 1998). In addition to anchoring the RII regulatory subunit of PKA, AKAP95 binds chromatin at mitosis (Collas *et al.*, 1999). It also recruits the condensin complex after nuclear envelope breakdown in mitotic HeLa cell extracts (Steen *et al.*, 2000). PKA binding to AKAP95 is dispensable for the chromosome-condensation activity of AKAP95, but maintenance of chromosomes in a condensed form throughout mitosis requires PKA activity and binding (Collas *et al.*, 1999). In this study, we mapped the domains of human AKAP95 involved in chromatin binding, condensin targeting and chromosome condensation in mitotic extract. We also present evidence for an interaction between

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This paper is dedicated to the memory of Dr Kathrine Le Guellec, who regrettably passed away while this paper was in preparation.

AKAP95 and a non-SMC subunit of the *Xenopus* condensin complex.

RESULTS

AKAP95 deletion and mutant proteins

We have shown that the C-terminal half of AKAP95 [AKAP95(387–692)] binds mitotic chromatin and is implicated in chromosome condensation and condensin recruitment (Steen *et al.*, 2000). Here, we investigated molecular determinants of chromatin-binding, condensin-binding and chromosome-condensation activities in the C-terminal domain of AKAP95. As a negative control, the first 195 N-terminal amino acids of AKAP95 [AKAP95(1–195)] were used. A panel of C-terminal deletions of AKAP95 was generated (Figure 1). Single or double mutations were also introduced into AKAP95(387–692) to abolish PKA binding (I582P) (Carr *et al.*, 1992) or to disrupt the structure of either ZF1 or ZF2 by mutating two of the zinc-chelating cysteines (C392S, C395S ‘ZF1CCSS’ in ZF1 and C481S, C484S ‘ZF2CCSS’ in ZF2) (Figure 1, asterisks).

Mapping of the chromatin-binding domain and chromosome-condensation activity of AKAP95

Binding of each GST–AKAP95 peptide (500 ng/ml) to decondensed AKAP95-free chromatin (Figure 2A, No AKAP95) in mitotic extracts was examined by immunofluorescence using anti-AKAP95 or anti-GST antibodies. All C-terminal non-mutated AKAP95 peptides tested bound chromatin, whereas AKAP95(1–195) did not (Figure 2A). Lack of detection of AKAP95(1–195) on chromatin was not due to the use of anti-GST antibodies, as chromatin-binding peptides were detected on chromosomes with these antibodies (data not shown). Binding to chromatin was maintained after mutation of either ZF1, ZF2 or the PKA-binding domain in AKAP95(387–692) (Figure 2A). However, mutation of ZF1 in AKAP95(387–450) abolished chromatin binding. Thus, amino acids 387–450 are sufficient for chromatin binding, and ZF1 is involved in this function. ZF2 appears to be dispensable for chromatin binding; however, it can substitute for ZF1 to mediate chromatin anchoring when the latter is non-functional.

To map the chromosome-condensation activity of AKAP95, AKAP95-free chromatin (see Methods) was incubated in mitotic extract containing 500 ng/ml of either AKAP95 peptide, and chromatin morphology was assessed after 1 h. Data are shown in Figure 2B and C. As expected, no chromatin condensation was observed in the absence of recombinant AKAP95 (row 1) or with peptides that do not bind chromatin (rows 3 and 11). Full-length AKAP95 (row 2), AKAP95(387–692), AKAP95(387–602), AKAP95(387–569) (rows 4–6) and AKAP95(387–692)I582P (row 12) supported chromosome condensation. In contrast, AKAP95(387–524) and AKAP95(387–450) did not promote condensation (rows 7 and 8). Likewise, disrupting ZF1 or ZF2 structure abolished condensation activity of AKAP95 (rows 9 and 10). Similar observations were made when looking at DNA labeling in Figure 2A. Therefore, chromosome condensation involves a critical domain within residues 525–569 in addition to the ZFs.

Fig. 1. GST–AKAP95 deletion and mutant proteins. Amino acid numbers are indicated. Asterisks indicate mutation(s) in the ZFs and the PKA-binding domain of AKAP95.

AKAP95-mediated recruitment of the condensin complex to chromosomes

We have shown previously that chromatin-bound AKAP95(387–692) recruits the condensin complex in mitotic HeLa cell extracts (Steen *et al.*, 2000). To define the domain of AKAP95 capable of targeting the condensin complex to chromosomes, AKAP95 peptides were allowed to bind to AKAP95-free chromatin and tested for their ability to recruit condensins. Condensin targeting was judged by purification of chromatin through sucrose and immunoblotting, using antibodies to all hCAP subunits. Data are shown in Figure 3. All condensin subunits were recruited to chromatin harboring full-length recombinant AKAP95 (lane 2), endogenous AKAP95 (lane 9) and AKAP95(387–692) (lane 4) but not to AKAP95-depleted chromatin (lane 1). AKAP95(1–195) did not promote condensin targeting (lane 3), as anticipated by its inability to bind chromatin. AKAP95(387–602) and AKAP95(387–569) recruited condensins (lanes 5 and 6), in contrast to AKAP95(387–524) and AKAP95(387–450) (lanes 7 and 8) despite their binding to chromatin (see Figure 2A). Moreover, mutation of the PKA-binding domain did not affect condensin targeting (lane 10). Interestingly, however, mutation of ZF2, but not ZF1, in AKAP95(387–692) abrogated condensin recruitment (lanes 11 and 12). Together with our previous data, this indicates that determinants necessary for condensin recruitment include ZF2 and residues 525–569 of AKAP95. Functional ZF1 is dispensable for condensin targeting but appears to be essential for chromosome condensation.

Human and *Xenopus* condensin subunits have recently been shown to be interchangeable in a cell-free *Xenopus* chromosome-condensation assay (Kimura *et al.*, 2001). In an attempt to determine any interaction between AKAP95 and condensins, AKAP95 was immunoprecipitated from HeLa chromatin condensed in a mitotic extract (Collas *et al.*, 1999). Figure 4A shows that AKAP95 coprecipitated a complex containing all hCAP subunits. To further examine association of AKAP95 with condensins, AKAP95 and XCAP-H were expressed in yeast as Gal4-BD or -AD fusion proteins, and diploids expressing both proteins were produced by mating. Figure 4B shows growth of colonies for diploids expressing both AKAP95 and XCAP-H that tested positive for β -galactosidase (β -gal) activity, indicating a possible interaction between AKAP95 and XCAP-H. This interaction

Fig. 2. Mapping of chromatin-binding domain and chromosome-condensation activity of AKAP95. Decondensed HeLa cell chromatin depleted of endogenous AKAP95 was exposed to mitotic extract containing 500 ng/ml of the indicated GST-AKAP95 peptides. As controls, chromatin harboring endogenous AKAP95 or AKAP95-depleted chromatin exposed to extract containing 500 ng GST only were used. (A) Binding of GST-AKAP95 peptides to chromatin was assessed by immunofluorescence using anti-AKAP95 mAb47. AKAP95(1-195) binding was detected with anti-GST antibodies. DNA was labeled with Hoechst 33342. (B) Proportions (mean $\pm$ SD) of chromatin masses undergoing condensation after 1 h in mitotic extract. (C) Phase contrast views of chromatin morphology upon incubation of AKAP95-free chromatin with peptides indicated in (B). Scale bars = 10 μ m.

was confirmed by coprecipitation of *in vitro* translated [35 S]Met-labeled full-length XCAP-H with GST-AKAP95(1-692) (200 nM) *in vitro* (Figure 4C). Note that GST-AKAP95 did not coprecipitate with an XCAP-H(329-367) fragment (data not shown). Moreover, full-length hCAP-D2 did not interact with AKAP95(387-692) in yeast, suggesting that association of AKAP95 with the condensin complex may involve at least the hCAP-H subunit (Figure 4D). Interaction of other condensin subunits with AKAP95 were not tested. Interestingly, hCAP-D2 was shown to interact with XCAP-H, as judged by both colony growth and β -gal activity (Figure 4D).

Rescue of premature chromatin decondensation by AKAP95

Incubation of condensed chromosomes in mitotic extract immunodepleted of soluble AKAP95 elicits premature chromosome decondensation, a phenomenon originally referred to as PCD (Collas *et al.*, 1999). Additional experiments have shown that condensed chromosomes exposed to cell lysis buffer used to prepare the extract do not undergo PCD (T. Eide and P. Collas, unpublished results), suggesting that PCD requires as yet unidentified mitotic cytosolic factors.

Fig. 3. Condensin recruitment to chromatin by AKAP95 in mitotic extract. Chromatin fractions harboring either no AKAP95 (lane 1), endogenous AKAP95 (lane 9) or indicated GST-AKAP95 peptides were purified by sedimentation through sucrose and immunoblotted using anti-hCAP-C, -D2, -E, -G and -H antibodies. Histone H4 was immunoblotted as a loading control.

Fig. 4. AKAP95 interacts with XCAP-H *in vivo* and *in vitro*. (A) AKAP95 was immunoprecipitated from HeLa cell chromatin condensed in mitotic extract. Immune and control (IgG) precipitates were immunoblotted using antibodies to all hCAP subunits. (B) Yeasts expressing full-length AKAP95 or XCAP-H were mated. Interaction of AKAP95 and XCAP-H was assessed by growth of diploids on triple drop-out (TDO) selection medium and β -gal activity. pAS2-1 and pGADGH vectors without inserts served as negative controls. (C) *In vitro* transcribed and translated [35 S]Met-labeled full-length XCAP-H (lane 1) was incubated with GST alone (lane 2) or 200 nM GST-AKAP95 (lane 3). XCAP-H coprecipitated with GST or GST-AKAP95 using glutathione beads was detected by autoradiography. (D) Yeasts expressing full-length hCAP-D2, XCAP-H or AKAP95(387–692) were mated. Interaction of hCAP-D2 with XCAP-H or AKAP95(387–692) was assessed by growth of diploids on quadruple drop-out (QDO) selection medium and β -gal activity. pAS2-1 and pACT2 vectors without inserts were used as negative controls.

The GST-AKAP95 peptides were tested for their ability to restore condensation of prematurely decondensed chromatin (Figure 5A). This was defined as the re-condensation of swollen and diffuse chromosome regions (characteristic of PCD) into highly compact structures (Figure 5B). HeLa nuclei condensed in mitotic extracts were exposed to a fresh AKAP95-depleted extract for 1 h to achieve PCD. AKAP95 peptides (500 ng/ml) were added, and chromosome morphology was evaluated after 1 h by phase contrast microscopy. Figure 5B shows input

PCD chromatin before peptide addition. AKAP95(1–692), AKAP95(387–692) and AKAP95(387–602) restored condensation of nearly 100% of chromosomes (Figure 5B and C). All other peptides tested did not rescue condensation (Figure 5B and C). Notably, mutation of ZF1, ZF2 or the PKA-binding domain abolished the ability of AKAP95(387–692) to restore condensation (Figure 5C). Thus, the AKAP95 domain responsible for restoring condensation of PCD chromatin lies within residues 387–602 and requires intact ZFs and a functional PKA-binding domain.

Fig. 5. Rescue of condensation of prematurely decondensed chromatin by the GST-AKAP95 peptides. **(A)** HeLa chromatin condensed in mitotic extract was exposed to AKAP95-depleted mitotic extract to induce PCD. Chromatin-binding GST-AKAP95 peptides were added to the extract, and restoration of condensation was monitored by phase contrast microscopy within 1 h. **(B)** Representations of input prematurely decondensed chromosomes (left), rescued chromosome condensation [middle, obtained with AKAP95(1-692)] and failure to restore condensation [right, obtained with AKAP95(387-569)]. Enlargements of framed areas are also shown. Scale bar = 10 μ m. **(C)** Summary of the effect of GST-AKAP95 peptides tested on rescue of chromosome condensation.

DISCUSSION

We have shown previously that chromosome-bound AKAP95 functions as a targeting protein for the condensin complex in a mitotic extract (Steen *et al.*, 2000). We have now mapped the chromatin-binding, condensin-binding and chromosome-condensation activities of AKAP95 in the C-terminal half of the protein and identified distinct requirements for each activity.

One chromatin-binding domain of AKAP95 lies within residues 387–450 and requires ZF1. This suggests that ZF1 mediates the binding of AKAP95 to DNA at least, as proposed for rat AKAP95 *in vitro* (Coghlan *et al.*, 1994). Curiously, ZF2 is dispensable for chromatin binding; however, it may substitute for ZF1 when the latter is rendered non-functional by mutation. Chromosome-condensation activity is conferred by several determinants within the region 387–569: (i) the chromatin-binding domain; (ii) a key element within residues 525–569; and (iii) both ZFs. Deletion or mutation of the PKA-binding domain of AKAP95 does not impair condensation, indicating that association of PKA with AKAP95 is dispensable for this activity. This supports our earlier findings that disruption of AKAP95–PKA anchoring does not affect chromosome condensation *in vitro* and *in vivo* (Collas *et al.*, 1999).

Residues 387–569 of AKAP95 are also sufficient to recruit condensins to chromosomes. Interestingly, either none or all hCAP subunits were recruited to chromatin, and no specific subunit was targeted by a particular AKAP95 peptide, suggesting recruitment of the condensin complex as a whole to chromosomes. Furthermore, disruption of ZF1 abrogates chromosome condensation but not condensin recruitment. Thus, ZF1 is essential for chromosome condensation independently of condensin recruitment. This suggests that condensin recruitment *per se* is not sufficient to drive condensation in the mitotic extract. Condensation may involve a stabilized interaction of condensins with AKAP95 or chromatin (e.g. via histone H3; Schmiesing *et al.*, 2000) mediated by ZF1. Mutating ZF2 abrogates condensin recruitment, as does the elimination of residues 525–569. Thus, it is possible that these residues are implicated in condensin binding by stabilizing the adjacent ZF2. We were not able to identify any distinct motif, such as HEAT repeats found in several proteins associated with condensins (Neuwald and Hirano, 2000), within residues 387–569 of AKAP95.

We present evidence for a direct interaction between AKAP95 and at least one subunit of the condensin complex, XCAP-H, the *Xenopus* homolog of the *Drosophila* barren gene product (Baht *et al.*, 1996). The high degree of homology between XCAP-H

and its human ortholog hCAP-H (Hirano *et al.*, 1997) and the similar composition of the *Xenopus* and human condensin complex (Schmiesing *et al.*, 2000; Kimura *et al.*, 2001) predict an interaction of AKAP95 with the human condensin complex via hCAP-H. The non-SMC components of the condensin complex have been proposed to function in the stimulation of SMC ATPase activity and the stable chromatin binding of condensins (Kimura and Hirano, 2000). Our findings support the latter hypothesis. AKAP95 does not seem to interact with hCAP-D2 in yeast; nevertheless, that AKAP95 associates directly with other condensin subunits is not excluded at present. Regardless, the detection of all hCAP subunits in AKAP95-bound chromatin and the co-immunoprecipitation of all hCAPs with AKAP95 from mitotic chromatin suggest that the entire 13S condensin complex is targeted to chromosomes.

The AKAP95 region responsible for maintaining chromosomes in a condensed form in the mitotic extract includes a functional PKA-binding domain, as shown by the failure to rescue PCD with an AKAP95(387–692) peptide harboring the I582P mutation. This supports our finding that disruption of PKA–AKAP95 anchoring or inhibition of PKA activity abrogates PCD rescue *in vitro* and in mitotic cells (Collas *et al.*, 1999). Why PKA anchoring to AKAP95 (and thereby in the vicinity of mitotic chromosomes) is required for maintenance of condensed chromosomes is unclear, but it may be explained by the involvement of PKA-dependent phosphorylation of chromatin substrates such as histone H3 (Van Hoover *et al.*, 1998; Schmiesing *et al.*, 2000).

The N-terminal half of AKAP95 may be implicated in other functions of AKAP95 within the interphase nucleus. Recently, a critical domain involved in targeting AKAP95 to the nuclear matrix was identified in the N-terminal portion of rat (and human) AKAP95, and a yeast two-hybrid screen revealed an interaction of amino acids 101–209 of rat AKAP95 with the p68 RNA helicase (Akileswaran *et al.*, 2001). Therefore, we propose that AKAP95 acts as a multivalent scaffolding protein, with different domains of the protein performing distinct roles during the cell cycle and in distinct subcellular compartments. This adds evidence to the growing concept that AKAPs assemble and integrate signals derived from multiple pathways (Felicciello *et al.*, 2001).

METHODS

Antibodies and peptides. Polyclonal antibodies and monoclonal mAb47 against AKAP95 (Coughlan *et al.*, 1994; Collas *et al.*, 1999) were from Upstate Biotechnology and Transduction Laboratories. Binding of these antibodies were mapped to residues 525–569 and 387–524, respectively (data not shown). Anti-NuMA monoclonal antibodies were from Transduction Laboratories. Anti-histone H4 antibodies were from Serotec and anti-GST antibodies from Santa Cruz Biotechnology. Rabbit polyclonal antibodies against hCAP-C, hCAP-H and hCAP-G (Kimura *et al.*, 2001) and hCAP-D2 (Collas *et al.*, 1999) were described previously. Anti-hCAP-E serum was made against residues 1183–1197 (AKSKAKPPKGAHEV) of hCAP-E. Full-length AKAP95 was amplified by PCR using AKAP95 cDNA (Eide *et al.*, 1998) as a template to generate restriction sites for subcloning into pGEX-KG and produce a GST–AKAP95(1–692) fusion protein. Expression of GST–AKAP95(387–692) was as described previously (Eide *et al.*, 1998). The two pGEX-AKAP95 expression

vectors were used as templates for site-directed mutagenesis (Quick Change, Stratagene) to generate a deletion/mutation panel of GST–AKAP95 peptides. These peptides were designated AKAP95(1–195), AKAP95(387–692)I582P, AKAP95(387–692)C392S,C395S [called AKAP95(387–692)ZF1CCSS], AKAP95(387–692)C481S,C484S [called AKAP95(387–692)ZF2CCSS], AKAP95(387–602), AKAP95(387–569), AKAP95(387–524), AKAP95(387–450) and AKAP95(387–450)C392S,C395S [called AKAP95(387–450)ZF1CCSS] (Figure 1). All constructs were sequenced, and expression of proteins was as described previously (Eide *et al.*, 1998).

Nuclei and chromatin. Nuclei were isolated by Dounce-homogenization of interphase HeLa cells (Collas *et al.*, 1999). To produce decondensed chromatin masses devoid of AKAP95, HeLa nuclei were loaded with anti-NuMA antibodies (1:40 dilution, 1 h), washed and exposed to a mitotic extract for 1 h (Steen *et al.*, 2000). Anti-NuMA antibodies promoted solubilization of the nuclear matrix protein NuMA together with AKAP95 and prevented association of AKAP95 with chromatin (Steen *et al.*, 2000). AKAP95-free decondensed chromatin masses were purified by sedimentation at 1000 g through 1 M sucrose for 10 min. For biochemical analyses, nuclei and chromatin were purified from mitotic extract by sedimentation through sucrose as above and dissolved in SDS sample buffer.

Mitotic extract and analysis of chromatin dynamics. HeLa cells synchronized in mitosis with 1 μ M nocodazole for 17 h (Eide *et al.*, 1998) were homogenized in hypotonic buffer (Collas *et al.*, 1999). The lysate was cleared at 10 000 g for 10 min and at 200 000 g for 3 h to produce a mitotic cytosolic extract. Nuclear breakdown and chromatin condensation were carried out at 30°C for up to 60 min in a mitotic extract containing an ATP-generating system (Collas *et al.*, 1999). Condensation was characterized by compaction of the chromatin and resolution into distinct chromosomes, as judged by DNA staining with 0.1 μ g/ml Hoechst 33342 and phase-contrast microscopy (Steen *et al.*, 2000). To produce prematurely decondensed chromosomes, HeLa chromatin condensed in a mitotic extract was recovered by sedimentation at 1000 g through 1 M sucrose, washed by resuspension and sedimentation in lysis buffer and incubated in fresh mitotic extract immunodepleted of AKAP95. PCD occurred within 2 h and referred to swelling of entire chromosomes or chromosome regions resulting in a ‘hazy’ chromosome morphology (Collas *et al.*, 1999; see Results). Immunoblotting, immunofluorescence and image analyses were performed as described previously (Collas *et al.*, 1999).

Yeast two-hybrid system. Full-length AKAP95 was cloned into the yeast vector pAS2-1. Full-length XCAP-H and hCAP-D2 (donated by K. Le Guellec, CNRS, University of Rennes, France) were cloned into pGADGH and pAS2-1, respectively (Matchmaker, Clontech). AKAP95(387–692) was cloned into pACT2. Constructs were transformed into two yeast strains of opposite mating type (PJ69–2A and Y187). Yeasts were grown in synthetic medium without Trp (pAS2-1) or without Leu (pGADGH and pACT2) to select for transformants. Interaction was tested by yeast mating. Diploid cells were grown on synthetic medium without Trp, Leu and His (triple drop-out, TDO) or on medium without Trp, His, Ade and Leu (quadruple drop-out, QDO). Growth on medium in the absence of His and Ade and β -gal activity analyzed by colony filter-lift assay were used as interaction markers.

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In vitro transcription and translation of XCAP-H. The T7 promoter was incorporated upstream of XCAP-H by PCR (Matchmaker Co-IP kit, Clontech). *In vitro* transcription and translation was performed using a TNT T7-coupled reticulate lysate system (Promega) and [³⁵S]Met labeling (Amersham). *In vitro* translated XCAP-H was analyzed by SDS-PAGE before use.

GST precipitation. ³⁵S-labeled full-length XCAP-H was mixed with 200 nM GST-AKAP95 in a buffer containing 50 mM Tris-HCl pH 7.4, 300 mM NaCl, 1 mM dithiothreitol, 1 mM PMSF, 0.1% Triton X-100, 1 mM EDTA, 5 mM benzamidine and protease inhibitors and incubated at room temperature for 30 min with rotation. Glutathione-agarose beads (25 µl) were added and incubated for 2 h at 4°C with rotation, after which beads were pelleted by centrifugation at 1000 g for 5 min. Beads were washed three times in 300 µl of the above buffer, and the precipitates were eluted by boiling in SDS sample buffer and subjected to SDS-PAGE. Precipitated XCAP-H was detected by autoradiography.

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