Role for Tyrosine Phosphorylation of A-kinase Anchoring Protein 8 (AKAP8) in Its Dissociation from Chromatin and the Nuclear Matrix

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Background: Tyrosine kinases are active in the cell nucleus and involved in global nuclear structure.

Results: Phosphorylation of AKAP8 at multiple tyrosine residues by several nucleus-localized tyrosine kinases, including c-Src, induces AKAP8’s dissociation from nuclear structures.

Conclusion: Nuclear tyrosine phosphorylation of AKAP8 is involved in global nuclear structure changes.

Significance: These findings highlight the importance of nuclear tyrosine phosphorylation in dynamic chromatin regulation.

Protein-tyrosine phosphorylation regulates a wide variety of cellular processes at the plasma membrane. Recently, we showed that nuclear tyrosine kinases induce global nuclear structure changes, which we called chromatin structural changes. However, the mechanisms are not fully understood. In this study we identify protein kinase A anchoring protein 8 (AKAP8/AKAP95), which associates with chromatin and the nuclear matrix, as a nuclear tyrosine-phosphorylated protein. Tyrosine phosphorylation of AKAP8 is induced by several tyrosine kinases, such as Src, Fyn, and c-Abl but not Syk. Nucleus-targeted Lyn and c-Src strongly dissociate AKAP8 from chromatin and the nuclear matrix in a kinase activity-dependent manner. The levels of tyrosine phosphorylation of AKAP8 are decreased by substitution of multiple tyrosine residues on AKAP8 into phenylalanine. Importantly, the phenylalanine mutations of AKAP8 inhibit its dissociation from nuclear structures, suggesting that the association/dissociation of AKAP8 with/from nuclear structures is regulated by its tyrosine phosphorylation. Furthermore, the phenylalanine mutations of AKAP8 suppress the levels of nuclear tyrosine kinase-induced chromatin structural changes. In contrast, AKAP8 knockdown increases the levels of chromatin structural changes. Intriguingly, stimulation with hydrogen peroxide induces chromatin structural changes accompanied by the dissociation of AKAP8 from nuclear structures. These results suggest that AKAP8 is involved in the regulation of chromatin structural changes through nuclear tyrosine phosphorylation.

The organization of global nuclear structure is drastically changed in a wide variety of conditions, such as growth factor stimulation, DNA damage responses, and tumorigenesis. Global nuclear structure changes, which we called chromatin structural changes (1, 2), are involved in regulating gene expression, DNA replication, DNA repair, mitotic progression, and so on (3–6).

Non-receptor-tyrosine kinases play important roles in cellular functions, including cell proliferation, adhesion, and differentiation (7, 8). Although tyrosine kinases mainly localize at the cytoplasm and the plasma membrane, they also localize in the nucleus to some extent (9). Src-family tyrosine kinases (SFKs), which are non-receptor-type tyrosine kinases, consist of proto-oncogene products and structurally related proteins, such as c-Src, Lyn, and Fyn (10, 11). We have shown that the members of SFKs localize in the nucleus and induce tyrosine-phosphorylation signals within the nucleus (1, 12–14). c-Abl, a non-receptor-type tyrosine kinase, shuttles between the cytoplasm and the nucleus (2, 15–17). ErbB4, a member of the ErbB family of receptor-tyrosine kinases, is cleaved upon ligand stimulation, and the intracellular domain is translocated into the nucleus (18, 19).

Despite the importance of non-receptor-type tyrosine kinases in signal transduction, most studies have focused on their roles as cytoplasmic signaling molecules (7, 8). Recently, nuclear tyrosine kinases were reported to be involved in the regulation of cytoskeletal structures, DNA damage responses, and gene transcription (20–22), although the roles of nuclear tyrosine kinases have not been fully understood. To investigate the role of nuclear tyrosine kinases in chromatin structural changes, we developed a quantita-

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The abbreviations used are: SFK, Src-family tyrosine kinase; AKAP8, A-kinase anchoring protein 8; KAP1, KRAB-associated protein 1; NLS, nuclear localization signal; TRITC, tetramethylrhodamine isothiocyanate; PI, propidium iodide; KD, kinase-dead.
Tyrosine Phosphorylation of AKAP8

To further understand the roles for nuclear tyrosine phosphorylation, we recently performed phosphoproteomic analysis of nuclear proteins in HeLa S3 cells expressing nucleus-targeted Lyn or c-Abl (nuclear localization signal (NLS)-fused Lyn and c-Abl). As a result, we identified various nuclear tyrosine-phosphorylated proteins, including protein kinase A-anchoring protein 8 (AKAP8/AKAP95). AKAP8 is a member of the large family of protein kinase A-anchoring proteins (AKAPs), which have a common function in binding to protein kinase A (23). AKAP8 was previously shown to be associated with nuclear structures, such as chromatin and the nuclear matrix (24–26). However, it is unclear whether the function of AKAP8 is regulated through a posttranslational modification.

In this study, we examined the roles of tyrosine phosphorylation of AKAP8 in nuclear tyrosine kinase-mediated chromatin structural changes. We showed that AKAP8 is one of the substrates for nuclear tyrosine kinases, and tyrosine phosphorylation of AKAP8 at multiple tyrosine residues inhibits its association with nuclear structures. We further showed that tyrosine phosphorylation of AKAP8 is involved in nuclear tyrosine kinase-mediated chromatin structural changes.

EXPERIMENTAL PROCEDURES

Plasmids—Expression vectors for intact c-Src, c-Src (c-Src-HA), and c-Src(KD) were constructed from cDNA encoding human wild-type Src (27) (provided by D. J. Fujita) as described (1, 28). Expression vectors for intact Lyn, NLS-Lyn, and NLS-Lyn-K275A (NLS-Lyn(KD)) were constructed from cDNA encoding human wild-type Lyn (29) (provided by T. Yamamoto) as described (1, 28). An expression vector for intact Fyn was constructed from cDNA encoding human wild-type Fyn (30) (provided by T. Yamamoto) as described (1, 28). An expression vector for NLS-c-Abl was constructed from cDNA encoding human wild-type c-Abl-1b (c-Abl-wt) (31) (provided by E. Canaani) as described (2). An expression vector for NLS-Syk was constructed from cDNA encoding human wild-type Syk (32) (provided by E. A. Clark) as described (1). An expression vector for NLS-I4CD (ErkB4 intracellular domain) was constructed from cDNA encoding human ErkB4 CYT-1 (33) (provided by S. Yokoyama) as described (19). An expression vector for wild-type AKAP8 tagged with an myc epitope at the N terminus (myc-AKAP8-wt) was constructed from cDNA encoding human wild-type AKAP8. cDNA that encode AKAP8 was generated by PCR from HeLa S3 cell cDNAs with 5′-CTGACGGTACCGCTGGTCTAATGGACAGGGCTACGGGCTACGGG-3′ (sense) and 5′-CTAGCTTCAGATCATCTTCTGGTGGAACAGCGTCTTTA-

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GACTCTGCA-3′ (antisense), and the BamHI-Xbal fragment of the PCR product was introduced into the BamHI-Xbal site of the pCDNA3/myc vector as described (34). The Tyr to Phe mutants of AKAP8 were created by PCR using AKAP8-wt as a template with primers as follows: Y51F/Y53F, 5′-GTACCACACA-

GCGATCACTTTCAGCTGGTCCCGACCGGTGG-3′ (sense) and 5′-GGCTGGGCGGCGAGTGAAGACTGCTC- GTGGTGCACTGGTGGTCTC-3′ (antisense); Y80F, 5′-TGC-

ATGCACTACGGCTAGGGACCCAGATGCGGGGC-3′ (sense) and 5′-GGCTGGGCGGCGAGTGAAGACTGCTC- GTGGTGCACTGGTGGTCTC-3′ (antisense); Y146F/Y150F/ Y152F, 5′-CAACACCCCTTCAGGCCTAGCTGCACG-

TTCGACTTTGAGTTCGACCTGG-3′ (sense) and 5′-GGCTGGGCGGCGAGTGAAGACTGCTC- GTGGTGCACTGGTGGTCTC-3′ (antisense); Y311F, 5′-GGAACGCAGGAAGTGCTTCTGGCAGGA-

GCCAGACACCAAACTG-3′ (sense) and 5′-GGCTGGTCTGG-

CTCTGGCAAGACGGTGAAGCTGGCTTCCGTTCTGCGC-

GG-3′ (antisense); Y436F, 5′-GGAGGTCTCTCAGGGAAT-

TCATTGAAACGAAGATIAGGATGGCTG-3′ (sense) and 5′-

CTTATTTCTGTTTCAATGAAATCTCGGAGAATCGC-

AGCTTCTGC-3′ (antisense); Y539F, 5′-GTAAAGATG-

CTGCAAAATTCTCCAGGATAGGACCCTTCCC-3′ (sense) and 5′-ACCCCTTTGAAAGATTCTCGGCACTTTC-

ACTATATGCTGTTG-3′ (antisense).

RNA Interference—Knockdown of AKAP8 was performed with short hairpin RNA (shRNA) for silencing AKAP8 (5′-GCCAGAGCCTTCTCCAA-3′) (26). The nucleotides for shRNA were annealed and subcloned into the BglIII-XbaI site of the pENTR4/H1 vector (provided by H. Miyoshi) (35, 36). The EBNA1-based episomal pEBMulti-H1 vector, which encodes the H1 promoter and a neomycin-resistant gene, was generated from the pEBMulti vector (Wako Pure Chemical Industries, Osaka, Japan) by replacing the CAG promoter with the H1 promoter as described (37). The oligonucleotides used for shRNA were annealed and subcloned into the pEBMulti-H1 vector (37–39). To generate AKAP8-knockdown cells, HCT116 cells were transfected with pEBMulti-neo/shAKAP8 selected in 600 µg/ml G418. Viable parental HCT116 cells were not detected after a 5-day selection using 600 µg/ml G418.

Antibodies—The following antibodies were used: phosphotyrosine (Tyr(P)) (4G10 and polyclonal antibody; Abcam or Santa Cruz Biotechnology), AKAP8 (Millipore; Abcam). Horseradish peroxidase-conjugated F(ab′)2 secondary antibodies were purchased from Amersham.
Biosciences. FITC- and TRITC-labeled IgG secondary antibodies and Alexa Fluor 488-, Alexa Fluor 546-, and Alexa Fluor 647-labeled IgG secondary antibodies were purchased from BioSource International, Sigma, and Invitrogen.

Cell Culture and Transfection—HeLa S3 cells (Japanese Collection of Research Bioresources, Osaka), COS-1 cells, and HCT116 cells were cultured in Iscove’s modified Dulbecco’s medium containing 1% fetal bovine serum and 4% bovine serum. Cells seeded in a 35-mm (60-mm) culture dish were transiently transfected with 1 µg (3 µg) of plasmid DNA using 5 µg (15 µg) of linear polyethylenimine (25 kDa) (Polyscience, Inc.) (41) or Lipofectamine 2000 (Invitrogen). To inhibit SFK-
mediated or c-Abl-mediated tyrosine phosphorylation, cells were treated with 10–20 μM PP2 (Sigma) or 10 μM imatinib (LC Laboratories). To induce oxidative stress, cells were treated with 1 mM H₂O₂ at 37 °C for 1 h in Iscove’s modified Dulbecco’s medium.

Western Blotting and Immunoprecipitation—Cell lysates were prepared in SDS-PAGE sample buffer or Triton X-100 lysis buffer (30 mM HEPES, pH 7.4, 100 mM NaCl, 0.5% Triton X-100, 4 mM EDTA, 10 mM NaF, 10 mM Na₃VO₄, 4 μg/ml aprotinin, 4 μg/ml leupeptin, 1.6 μg/ml pepstatin A, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and subjected to SDS-PAGE and electrotransferred onto PVDF membranes (Millipore). Immunodetection was performed by enhanced chemiluminescence (Millipore) as described (38, 42). Results were analyzed using a ChemiDoc XRS-Plus image analyzer (Bio-Rad). Immunoprecipitation was performed using antibody-precoated protein-G beads as described (13, 36). The intensity of chemiluminescence was measured using Quantity One software (Bio-Rad). Bars represent the means ± S.D. from three independent experiments. Asterisks indicate significant differences (*, p < 0.05; **, p < 0.01) calculated by Student’s t test.

Tyrosine Phosphorylation of AKAP8—To identify the tyrosine-phosphorylated proteins in the nucleus, we established cell lines that express either Lyn tyrosine kinase-tagged with a nuclear localization signal (NLS-Lyn) or c-Abl tyrosine kinase tagged with a nuclear localization signal (NLS-c-Abl). Nuclear tyrosine-phosphorylated proteins were purified with anti-Tyr(P) antibody as recently reported (14, 16, 34). We

Western Blotting analysis was performed with anti-myc and anti-Tyr(P) antibodies. Western blotting analysis was performed with anti-AKAP8 and anti-actin antibodies. Relative amounts of AKAP8 were quantitated.
Tyrosine Phosphorylation of AKAP8

A) myc-AKAP8-wt

B) HeLa S3

C) HeLa S3

D) HeLa S3

E) COS-1
identified the nuclear structure-binding protein AKAP8 as a candidate substrate of nuclear tyrosine kinases. To validate tyrosine phosphorylation of AKAP8, we cotransfected cells with myc-tagged AKAP8 (myc-AKAP8-wt) plus NLS-Lyn or myc-AKAP8-wt plus NLS-Lyn(KD) in the presence or absence of the SFK inhibitor PP2 and subjected to immunoprecipitation and Western blotting analysis. myc-AKAP8-wt was tyrosine-phosphorylated by NLS-Lyn but not NLS-Lyn(KD), and PP2 treatment inhibited tyrosine phosphorylation of myc-AKAP8-wt (Fig. 1A). Next, to examine which SFK members dominantly phosphorylate AKAP8, we cotransfected cells with myc-AKAP-wt plus intact c-Src, intact Lyn, or intact Fyn. Expression of c-Src was found to strongly induce tyrosine phosphorylation of AKAP8 among SFKs (Fig. 1B). Our previous studies showed that several tyrosine kinases, including SFKs and c-Abl, except for Syk, located in the nucleus are involved in induction of chromatin structural changes (1, 2, 19). Interestingly, AKAP8 was tyrosine-phosphorylated by not only NLS-Lyn but also NLS-c-Abl and NLS-4ICD but was not by NLS-Syk (Fig. 1C). These results suggest that AKAP8 is tyrosine-phosphorylated by some nuclear tyrosine kinases such as SFKs, c-Abl, and 4ICD.

**Effect of Tyrosine Phosphorylation of AKAP8 on Its Association with Nuclear Structures**—To elucidate the role of tyrosine phosphorylation in AKAP8 localization, we examined whether NLS-Lyn expression affected the localization of AKAP8 in the nucleus. Expression of NLS-Lyn diffusely delocalized endogenous AKAP8 in the nucleus, whereas endogenous AKAP8 in control cells appeared to be localized to some nuclear structures, suggesting that NLS-Lyn expression changes the localization of AKAP8 in the nucleus (Fig. 2A). Notably, neither expression of NLS-Lyn(KD) nor inhibition of NLS-Lyn by PP2 induced delocalization of AKAP8 (Fig. 2A). Considering that AKAP8 is associated with the nuclear matrix and chromatin (24), these results raise the intriguing possibility that tyrosine phosphorylation of AKAP8 is capable of dissociating AKAP8 from nuclear structures, e.g., the nuclear matrix and chromatin.

To examine the effect of tyrosine phosphorylation of AKAP8 on its association with nuclear structures, cells were transfected with NLS-Lyn, extracted with 0.2% Triton X-100 extraction buffer, and stained for AKAP8. Without extraction, the levels of AKAP8 were not changed irrespective of NLS-Lyn expression. Intriguingly, the levels of Triton X-100-insoluble AKAP8 were drastically decreased in NLS-Lyn-expressing cells compared with control cells (Fig. 2B). Expression of wild-type c-Src, which is present in the nucleus, increased the level of AKAP8 (Fig. 2C). These results suggest that nuclear SFKs are involved in dissociation of AKAP8 from nuclear structures. To further ascertain the effect of tyrosine phosphorylation on AKAP8 dissociation from nuclear structures, cells were subfractionated into Triton X-100-soluble and insoluble fractions. Triton-X-100-insoluble fraction contained the chromatin protein histone H3 and the nuclear lamina protein lamin A/C (Fig. 2D). Western blotting analyses showed that the amounts of endogenous AKAP8 present in insoluble fraction were decreased upon NLS-Lyn expression (Fig. 2, D and E). However, NLS-Lyn(KD) expression did not decrease the amounts of endogenous AKAP8 in insoluble fraction, and PP2 treatment inhibited the decrease in the amounts of endogenous AKAP8 present in insoluble fraction upon NLS-Lyn expression (Fig. 2E).

Next, we cotransfected cells with myc-AKAP8-wt plus NLS-Lyn, subsequently extracted with 0.2% Triton X-100 extraction buffer, and stained for AKAP8. Consistent with endogenous AKAP8 (Fig. 2B), the levels of Triton X-100-insoluble myc-AKAP8-wt were drastically decreased upon NLS-Lyn expression (Fig. 2F). Subcellular fractionation experiments showed that expression of NLS-Lyn or c-Src decreased the amounts of myc-AKAP8-wt in Triton X-100-insoluble fraction in a kinase activity-dependent manner (Fig. 2, G and H). Importantly, the tyrosine phosphorylation levels of myc-AKAP8-wt in Triton X-100-soluble fraction were much higher than those in Triton X-100-insoluble fraction (Fig. 2F). Furthermore, we examined whether endogenous SFK activity affected the association of endogenous AKAP8 with nuclear structures and found that treatment of HCT116 cells, which highly express endogenous c-Src, with PP2 increased the amounts of AKAP8 in Triton X-100-insoluble fraction compared with that with control or the Abl inhibitor imatinib (Fig. 2F). Taken together, these results indicate that tyrosine phosphorylation of AKAP8 by SFKs in the nucleus is capable of dissociating AKAP8 from nuclear structures.

**Dissociation of AKAP8 from Nuclear Structures through Phosphorylation of Its Multiple Tyrosine Residues**—We detected tyrosine-phosphorylated peptides of AKAP8-Tyr(P)-311 (YQQLpYEEDPTK) and AKAP8-Tyr(P)-436 (LPDKTCEFLQEpYIVNR) by mass spectrometry. In the PhosphoSitePlus proteomics database, phosphorylation of AKAP8 at Tyr-150, Tyr-154, Tyr-170, and Tyr-311 are listed (44). A previous phosphoproteomic study showed that Tyr-152 is phosphorylated upon PDGF stimulation (45). Taken together, we speculated that AKAP8 might be phosphorylated at multiple tyrosine residues by nuclear tyrosine kinases.

To identify tyrosine phosphorylation sites of AKAP8 that could affect the localization of AKAP8, we constructed several YF mutants that were mutated tyrosine to phenylalanine (Fig. 3A). We then cotransfected cells with myc-AKAP8-wt plus NLS-Lyn or its YF mutant plus NLS-Lyn, extracted with 0.2% Triton X-100 extraction buffer, and stained for myc-AKAP8-wt

![FIGURE 3. Effect of tyrosine phosphorylation of AKAP8 on its dissociation from nuclear structures.](image-url) A schematic representations of AKAP8 and its mutants. **Gray bars** indicate the sites of tyrosine residues on AKAP8. **Black bars** indicate the sites of tyrosine residues that are mutated to phenylalanine. **NMTS**, nuclear matrix-targeting sequence; **ZF**, zinc finger motif; **PKA**, protein kinase A-binding domain. B, C, and D, HeLa S3 cells cotransfected with myc-AKAP8-wt plus vector or NLS-Lyn or with its mutant plus vector or NLS-Lyn were cultured for 24 h. Cells were extracted with 0.2% Triton X-100 extraction buffer, fixed, and stained with anti-myc and anti-Tyr(P) antibodies. Note that cells expressing NLS-Lyn could be detected with anti-Tyr(P) antibody. The plot represents the mean intensity of anti-myc staining in each cell. E, COS-1 cells cotransfected with NLS-Lyn plus myc-AKAP8-wt or myc-AKAP8-YF were cultured for 24 h. myc-AKAP8-wt and myc-AKAP8-YF mutants were immunoprecipitated with anti-myc antibody. Western blotting analysis was performed with the indicated antibodies. Levels of tyrosine phosphorylation of myc-AKAP8-YF mutants are expressed as values relative to that of myc-AKAP8-wt after normalization with protein levels of myc-AKAP8-wt and its YF mutants.
or -mutant. 4CYF mutations (four tyrosine to phenylalanine mutations in the C terminus region; Y170F, Y311F, Y436F, and Y539F), including the tyrosine phosphorylation sites that we detected, did not inhibit dissociation of AKAP8 from nuclear structures (Fig. 3B). However, 5 NYF mutations (five tyrosine to phenylalanine mutations in the N terminus region; Y146F, Y150F, Y152F, Y154F, and Y170F) and 8 NYF mutations (eight tyrosine to phenylalanine mutations in the N terminus region; Y151F, Y53F, Y80F, Y146F, Y150F, Y152F, Y154F, and Y170F) only partially inhibited dissociation of AKAP8 from nuclear structures upon NLS-Lyn expression (Fig. 3, C and D). Notably, we found that 11 YF mutations (Y151F, Y153F, Y80F, Y146F, Y150F, Y152F, Y154F, and Y170F) strongly inhibited dissociation of AKAP8 from nuclear structures upon NLS-Lyn expression (Fig. 4, A–C).

Next, to examine whether these tyrosine residues are indeed tyrosine phosphorylation sites of AKAP8, we cotransfected cells with myc-AKAP8-wt plus NLS-Lyn or its YF mutants plus NLS-Lyn. NLS-Lyn-induced tyrosine phosphorylation levels of myc-AKAP8–4CYF, -5NYF, and -8NYF were partially decreased compared with myc-AKAP8-wt (Fig. 3E). Nevertheless, NLS-Lyn-induced tyrosine phosphorylation levels of myc-AKAP8–11YF were decreased to 44% that of myc-AKAP8-wt (Fig. 4D). Similar results were obtained by cotransfection with myc-AKAP8–11YF plus c-Src (Fig. 4E). Taken together, these results suggest that AKAP8 can dissociate from nuclear structures when its multiple tyrosine residues are phosphorylated by nuclear tyrosine kinases.

**Involvement of Tyrosine-phosphorylated AKAP8 in Chromatin Structural Changes**—We previously showed that SFKs induce chromatin structural changes (1). Our pixel imaging method showed a positive correlation between the S.D. values of PI fluorescence intensity and the levels of chromatin structural changes (1, 2). Thus, we examined whether there was a relationship between the S.D. values of PI fluorescence intensity of chromatin and the levels of AKAP8 association with nuclear structures. Consistent with our previous study (1), NLS-Lyn induced strong chromatin structural changes compared with control vector and NLS-Lyn(KD) (Fig. 5A). Two-dimensional-plot analyses showed that there was a strong inverse correlation between the levels of Triton X-100-insoluble AKAP8 and chromatin structural changes in NLS-Lyn-expressing cells but not NLS-Lyn(KD)–expressing cells (Fig. 5A). Similar to NLS-Lyn, the levels of Triton X-100-insoluble AKAP8 inversely correlated with chromatin structural changes in c-Src–expressing cells but not c-Src(KD)–expressing cells (Fig. 5B). These results indicate that the dissociation of AKAP8 from nuclear structures is positively correlated with nuclear tyrosine phosphorylation-mediated chromatin structural changes.

To examine whether the dissociation of AKAP8 from nuclear structures induced by nuclear tyrosine kinases is involved in tyrosine phosphorylation-mediated chromatin structural changes, we cotransfected cells with NLS-Lyn plus myc-AKAP8-wt or NLS-Lyn plus myc-AKAP8–11YF. The levels of chromatin structural changes induced by NLS-Lyn in myc-AKAP8–11YF–expressing cells were significantly lower than those in myc-AKAP8-wt–expressing cells (Fig. 6A). Furthermore, we examined the effect of AKAP8 knockdown on chromatin structural changes. The levels of chromatin structural changes in AKAP8-knockdown cells were higher than those in control cells (Fig. 6, B and C). These results suggest that the dissociation of AKAP8 from nuclear structures is involved in chromatin structural changes induced by nuclear tyrosine phosphorylation.

Oxidative stress activates tyrosine kinases, including SFKs (46–48), and affects gene transcription, epigenetic regulation, and regulation of global nuclear structure (49, 50). Also, we showed that treatment with H2O2 induces activation of SFKs and chromatin structural changes (1, 51). Therefore, we examined the effect of oxidative stress on the association of AKAP8 with nuclear structures. Consistent with our previous study (1), H2O2 treatment induced chromatin structural changes, which were inhibited in part by PP2 treatment (Fig. 6D). Importantly, by examining Triton X-100-insoluble fractions, we found that H2O2 treatment induced the dissociation of endogenous AKAP8 from nuclear structures, and its dissociation was inhibited in part by PP2 treatment (Fig. 6, D and E). These results suggest that oxidative stress-induced chromatin structural changes involve the dissociation of endogenous AKAP8 from nuclear structures through SFK activation.

**DISCUSSION**

Although AKAP8 is known to associate with nuclear structures such as the nuclear matrix and chromatin and act as a scaffolding protein (24–26), it has thus far not been reported that the interaction of AKAP8 with nuclear structures is regulated through posttranslational modifications. In the present study we show that AKAP8 can dissociate from nuclear structures through its tyrosine phosphorylation mediated by nuclear c-Src and that the dissociation of AKAP8 is involved in global nuclear structure changes, which we called chromatin structural changes (1, 2).

Many studies have shown the functions of serine/threonine kinases present in the nucleus (4, 6, 52). However, a limited...
FIGURE 5. Correlation between SFK-induced dissociation of AKAP8 and chromatin structural changes. A and B, HeLa S3 cells transfected with the indicated plasmids were cultured for 24 h. Cells were extracted and fixed with PTEMF buffer and subsequently stained with anti-HA and anti-AKAP8 antibodies and PI. Two-dimensional-plot analyses were performed with mean fluorescence intensities of anti-AKAP8 staining (vertical axis) versus S.D. values of PI intensity (horizontal axis) in individual cells, as described under “Experimental procedures.” n, cell number; r, regression coefficient.
number of studies showed the functions of the tyrosine kinases present in the nucleus (1, 2, 12, 14, 22, 53, 54). Because tyrosine kinases are largely located at the plasma membrane and the cytoplasm, tyrosine phosphorylation signals have been hardly detected in the nucleus. Nonetheless, carefully using a high dose of the potent tyrosine phosphatase inhibitor NaF/VO4, we were eventually able to detect tyrosine phosphorylation of nuclear proteins (1, 2, 12–14, 16, 19, 34, 53, 55). Given that tyrosine phosphatases are abundant in the nucleus (9), we assume that, like ON/OFF switching in a microprocessor, phosphorylation levels of nuclear proteins may be very rapidly regulated by a balance between the activity of nuclear tyrosine kinases and that of nuclear tyrosine phosphatases (1, 14, 34). To understand the role of nuclear tyrosine phosphorylation, we created nucleus-targeted tyrosine kinases and transfected them into cells. As a result, many unidentified nuclear proteins were found to be tyrosine-phosphorylated (1, 2). Our recent study showed that tyrosine phosphorylation of AKP1, which is one of the nuclear substrates that we found, induces the dissociation of CAP1 and heterochromatin protein 1α (HP1α) from heterochromatin (14). Our findings in this study also show that tyrosine phosphorylation of AKAP8, another nuclear protein, induces its dissociation from nuclear structures (Figs. 1–4). Collectively, some of the tyrosine kinases that are present in the nucleus may be involved in the regulation of the interaction of their nuclear substrates with chromatin and the nuclear matrix through tyrosine phosphorylation of their nuclear substrates per se.

We have been studying the functional commonalities and differences among nuclear tyrosine kinases (1, 2, 12, 14, 19, 53). Our results showed that NLS-Lyn induces tyrosine phosphorylation of AKAP8 much stronger than intact Lyn (Fig. 1, A and B), suggesting that nuclear Lyn is important for tyrosine phosphorylation of AKAP8. In contrast to Lyn, intact c-Src strongly induces phosphorylation of AKAP8 at tyrosine residues (Fig. 1B). Considering that c-Src induces high levels of phosphorylation of nuclear proteins, including CAP1, compared with the other SFK members (14), it is likely that, among SFKs, c-Src in particular plays an important role in tyrosine phosphorylation signaling in the nucleus. Alternatively, it is conceivable that the levels of tyrosine phosphorylation of the nuclear SFK substrates are attributable to the substrate specificity of individual SFKs, as Fyn induces tyrosine phosphorylation of AKAP8 but not CAP1 (Fig. 1B; see also Kubota et al. (14)).

Previous studies showed that the nuclear matrix-targeted sequence (amino acids 110–140) on AKAP8 is determined for the association of AKAP8 with the nuclear matrix (24). AKAP8 has also been shown to be recruited to mitotic chromosomes via the residues 387–450, including the zinc finger domain, and to play a role in mitotic progression (25, 26). Because the phosphorylation sites of AKAP8 at Tyr-51, -53, -80, -146, -150, -152, -154, and -170 are located close to the nuclear matrix-targeted sequence and those at Tyr-311, -436, and -527 are located close to the zinc finger domain (Figs. 3A and 4A), we hypothesize that phosphorylation at those tyrosine residues may interfere with the association of AKAP8 with the nuclear matrix and chromatin. In addition, AKAP8 is known to associate with the histone modification enzyme MLL2 complex via the N-terminal region of AKAP8 and regulate transcription in embryonic stem cells (56). Phosphorylation of AKAP8 at Tyr-51, -53, and -80 might be involved in MLL2-driven transcription in embryonic stem cells, as we showed that tyrosine phosphorylation-mediated chromatin structural changes lead to histone modifications and gene transcription (2, 19). Generation of AKAP8-YF knock-in embryonic stem cells might enable us to investigate an in vivo role of tyrosine phosphorylation of AKAP8 in transcription during development.

Chromatin structural changes are involved in gene expression, DNA replication, mitotic progression, and so on (1, 3–5). It is evident that AKAP8 knockdown significantly induces chromatin structural changes (Fig. 6, B and C), although the levels of chromatin structural changes were relatively low upon AKAP8 knockdown compared with those induced by expression of nuclear tyrosine kinases. Furthermore, oxidative stress induces chromatin structural changes, at least in part, by dissociating AKAP8 from nuclear structures through SFK activation (Fig. 6D). In addition, the association of AKAP8 with nuclear structures is involved in gene expression, DNA replication, and mitotic progression (26, 56, 57). Recently, the nuclear proteins KAT5, histone H4, and HDAC2 were reported to be tyrosine-phosphorylated and to functionally associate with chromatin structures (22, 54, 58). Taken together, we assume that tyrosine phosphorylation of various nuclear proteins, including AKAP8, is attributable to nuclear tyrosine kinase-mediated chromatin structural changes, which could be involved in a variety of nuclear functions, such as gene expression, DNA replication, and mitotic progression.

In conclusion, we identify AKAP8 as a nuclear substrate of nuclear tyrosine kinases. Although tyrosine phosphorylation of AKAP8 has been already reported, this is a first report of functional regulation of AKAP8 through its tyrosine phosphorylation. Multiple tyrosine phosphorylation of AKAP8 is involved

FIGURE 6. Involvement of AKAP8 in chromatin structural changes. A, HeLa S3 cells cotransfected with myc-AKAP8-wt plus vector or NLS-Lyn or with myc-AKAP8-YF plus vector or NLS-Lyn were cultured for 24 h. Cells were extracted with 0.2% Triton X-100 extraction buffer and subsequently fixed and stained with anti-Tyr(P) and anti-myc antibodies and PI. Note that cells expressing NLS-Lyn could be detected with anti-Tyr(P) antibody. The levels of chromatin structural changes were assessed using S.D. values of PI intensity per pixel. The plot represents the S.D. value of PI intensity per pixel in each cell from a representative experiment. The levels of chromatin structural changes were assessed as described in A. **, p < 0.01, D, COS-1 cells were pretreated with 20 μM PP2 for 2 h followed by treatment with PBS or 1 mM H2O2 for the last 1 h. Cells were extracted with 0.2% Triton X-100 extraction buffer and subsequently fixed and stained with the anti-AKAP8 antibody and PI. The levels of chromatin structural changes were assessed as described in A. **, p < 0.01. C, COS-1 cells were pretreated with 20 μM PP2 for 2 h followed by treatment with PBS or 1 mM H2O2 for the last 1 h. Cells were subjected to subcellular fractionation with 0.2% Triton X-100 extraction buffer. Western blotting analysis was performed with anti-AKAP8 and anti-actin antibodies. Amounts of endogenous AKAP8 in 0.2% Triton X-100-insoluble fraction are expressed as values relative to that in after normalization with actin levels.
in its dissociation from nuclear structures, leading to chromatin structural changes. Further studies will help us to deeply understand the relationship between nuclear tyrosine phosphorylation and chromatin structure.

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