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The KKRKK Sequence is Involved in Heat Shock-Induced Nuclear Translocation of the 18-kDa Actin-Binding Protein, Cofilin

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ABSTRACT. **The exposure of cultured mammalian cells to elevated temperatures induces the translocation of actin and cofilin into the nuclei and the formation of intranuclear bundles of actin filaments decorated by cofilin (actin/cofilin rods). Cofilin has a stretch of five basic amino acids, KKRKK, which was assumed to be the sequence involved in the heat shock-dependent accumulation of cofilin in nuclei. To examine this possibility, the site-directed mutagenesis technique was employed to alter the KKRKK sequence of cofilin to KTLKK and the mutated cofilin was expressed under the human β-actin promoter in transfectants of mouse C3H-2K cell line. All the recombinants derived from porcine cofilin cDNA were constructed so as to possess an extra-nonapeptide at their N-termini when expressed; their intracellular distribution could, therefore, be discriminated from that of endogenous cofilin using the indirect immunofluorescence method with polyclonal antibodies directed against the extra-peptide. The results clearly showed that the mutated cofilin possessing KTLKK instead of KKRKK did not translocate into the nuclei in response to heat shock whereas a recombinant cofilin with the unaltered sequence of KKRKK responded to heat shock and formed intranuclear rods together with actin. Although *in vitro* actin binding experiments showed that KTLKK-cofilin has a weaker affinity to actin filaments than KKRKK-cofilin, KTLKK-cofilin was found to form cytoplasmic actin/cofilin rods when transformants were incubated in NaCl buffer. Furthermore, we have noted that endogenous cofilin present in cells expressing KTLKK-cofilin behaved normally, translocated into nuclei and formed intranuclear actin/cofilin rods upon heat shock. These results suggest that the KKRKK sequence of cofilin functions as a nuclear location signal upon heat shock.**

Reorganization of cytoskeletal structures is one of the prominent responses to heat shock revealed by cultured mammalian cells (26). Among them, the most intriguing is the translocation of actin from cytoplasm to nuclei followed by the formation of intranuclear actin rods (8, 29). This phenomenon appeared to be similar to those induced by dimethyl sulfoxide (5) or cytochalasin D (31). Electron microscopic observations have revealed that the rods are bundles of thin filaments to which heavy meromyosin is bound (5, 31). We have found, however, that the actin rods in formaldehyde-fixed cells are not stained with fluorescent phalloidin (17). This observation was attributable to the fact that cofilin, an actin-binding protein, preoccupied phalloidin-binding sites on actin molecules, forming actin rods (17).

Cofilin is a low molecular weight actin-binding protein originally isolated from porcine brain (16). In the mixtures of purified actin and cofilin, cofilin binds to filamentous actin at pHs lower than 7.3 in a 1 : 1 molar ratio of cofilin to actin molecule in the filament, and depolymerizes actin filaments at pHs higher than 7.3 (24). cDNA clones encoding porcine (13), mouse (14), chicken (1) and human (18) cofilin have been cloned, revealing that the primary sequence of cofilin was highly conserved during evolution. Cofilin of these species consists of 166 amino acids residues and contains a KKRKK sequence which is similar to the nuclear location signal (NLS) sequence of SV40 large T antigen (9). This similarity is intriguing because cofilin is uniformly distributed throughout the cell space including the cytoplasm and nucleus, but is induced to accumulate in the nucleus upon heat shock (17). Considering the facts that actin does not have a NLS-like sequence (27, 28) and that cofilin forms a stoichiometrical complex with actin (16), we have suggested the possibility that cofilin forms a complex with actin and translocates into the nuclei upon heat shock (17). In this investigation, we attempted to examine the possibility of whether or not the KKRKK sequence of cofilin is a NLS which functionally operates upon heat shock.

Abbreviations used: NLS, nuclear location signal; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; Amino acids were conventionally expressed as single letters.

MATERIALS AND METHODS

Site-directed mutagenesis. pUcof9x contains the cDNA sequence encoding porcine cofilin with an additional N-terminal extra-nonapeptide (15, 33). To prepare a single-stranded DNA for *in vitro* site-directed mutagenesis, pTcof9x was constructed from a 2.4 kb *Sca*I-*BamH*I fragment of pUcof9x and a 1.4 kb *BamH*I-*Sca*I fragment of pTV118N (Takara Shuzo, Co., Kyoto). Site-directed mutagenesis was carried out using an *in vitro* mutagenesis kit from Amersham Japan, Co., Tokyo. The mutated sequences were confirmed by DNA sequencing of the dideoxy chain termination methods (23) using a sequence kit of United States Biochemical (Cleveland, Ohio).

Construction of mammalian expression plasmids for cofilin. There is an *Acc*III site at 21 bases downstream from the initiation codon within the sequence encoding the extra-peptide (15) but no usable restriction site in the 5′-noncoding region. For this reason, a double-stranded oligonucleotides containing an *Xba*I site at the 5′ end of the coding strand was synthesized which corresponds to a short stretch of the cDNA from the initiation codon to the *Acc*III site. Mammalian expression plasmids for cofilin were constructed by insertion of a 0.6 kb *Acc*III-*BamH*I fragment of pTcof9x and the synthetic oligonucleotides between *Xba*I and *BamH*I sites of pSVL, a mammalian expression vector with the SV40 late promoter (Pharmacia Japan, Co., Tokyo). A 1.2 kb *Xho*I-*Sal*I fragment of this plasmid was then inserted into the *Sal*I site of pHβApr-1, another mammalian expression vector with a human β-actin promoter. The resultant plasmid, pAcof9x, was used to express recombinant porcine cofilin in mouse C3H-2K cells.

Cells and transfection. Mouse fibroblastic cell line, C3H-2K, was described previously (7). Transfection was carried out according to the modified $CaPO_4$ method of Chen and Okayama (2) with the mammalian transfection kit from Strategene (La Jolla, CA). C3H-2K cells grown in a 60 mm dish were co-transfected with 15 μg pAcof9x and 1.5 μg pMAMneo (Clontech, Palo Alto, CA), a mammalian expression vector with a neomycin-resistant gene. Stable transformants were selected in the presence of 0.6 mg/ml G418, and cloned twice.

Induction of actin/cofilin rods. Cells to be tested were grown on 14 mm-ϕ glass coverslips contained in 24-well culture plates. To induce intranuclear actin/cofilin rods by an exposure to heat shock, the growth media, DMEM-10%FCS with or without G418, were replaced by Hepes-buffered DMEM containing 10%FCS. Twenty four-well culture plates were sealed with plastic tape and placed in a water bath prewarmed at the indicated temperatures. Cells were then incubated at 43.0°C (the temperature of medium in wells; 43.0± 0.3°C) for 60 min, and fixed in 3.7% formaldehyde/PBS. To induce cytoplasmic actin/cofilin rods, cells were incubated in a NaCl buffer at 37°C for 60 min as described previously (7).

Immunofluorescence staining. All recombinant cofilin expressed in C3H-2K cells had an extra-nonapeptide sequence, TMITPSSGN (15). Antibodies were raised against this peptide in rabbits and specific antibodies were purified using an affinity chromatography (33) (Fig. 1). An anti-actin antiserum was produced in a rabbit using chicken gizzard actin as an antigen (7). IgG fractions were prepared from the anti-actin serum and conjugated with fluorescein isothiocyanate (FITC) as described elsewhere (30). Procedures for immunofluorescence staining of cultured cells were described previously (7). Briefly, cells were fixed in 3.7% formaldehyde/PBS at room temperature for 20 min, and treated with 0.2% (w/v) Triton X-100 in PBS for 2 min. To determine the distribution of recombinant cofilin expressed, the fixed and permeabilized cells were sequentially incubated with anti-peptide antibodies and rhodamine-conjugated goat anti-rabbit IgG antibodies (Rh-G-anti-RIg). A double immunofluorescence method was used to determine the distribution of actin and recombinant cofilin in the same cells. The cells that had been stained with anti-peptide antibodies for recombinant cofilin were then incubated with FITC-labeled anti-actin antibodies in the presence of an excess amount of normal rabbit IgG (100 μg/ml).

Assay for binding of recombinant cofilin to actin filaments. [^{35}S]methionine-labeled recombinant cofilin was synthesized *in vitro* by the method of Zubay (34) with a prokaryotic DNA-directed translation kit from Amersham Japan, Co. (Tokyo). After incubation, reaction mixtures were centrifuged at 100,000 × g for 40 min and supernatant fractions were collected. An aliquot of each supernatant was mixed with purified rabbit muscle actin (0.2 mg/ml) and purified, unlabeled recombinant porcine KKRKK-cofilin (0.1 mg/ml) under actin polymerization conditions (100 mM KCl, 2 mM $MgCl_2$, 20 mM MES, pH 6.8), and incubated for 1 h at room temperature. Rabbit skeletal muscle actin and recombinant porcine cofilin were purified as described previously (15). After centrifugation at 100,000 × g for 40 min, pellets and supernatant fractions were resolved in SDS-PAGE (10) fol-

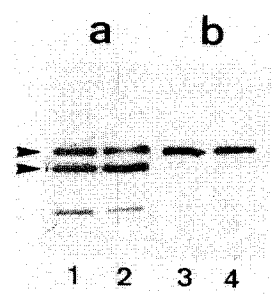

Fig. 1. Immune blots of total cell extracts from A2-21 and A3-91 cells with anti-cofilin antibodies and anti-peptide antibodies.
Total cell extracts prepared from A2-21 (lane 1 and 3) and from A3-91 (lane 2 and 4) were resolved by SDS-PAGE, and transferred to PVBD membrane (Millipore Co., Bedford, MA). *a.* rabbit anti-cofilin antibodies (17), *b.* rabbit anti-peptide antibodies (33) were used. Procedures for immune blot were described elsewhere (33). The upper and lower arrow heads indicate bands of recombinant porcine cofilin and those of endogenous mouse cofilin, respectively.

lowed by fluorography.

RESULTS

Polyclonal rabbit antibodies raised against porcine cofilin (17) bind equally to both porcine and mouse cofilin because these two cofilin polypeptides differ from each other in only two amino acid residues among the total 166 amino acid sequence (13, 14). To immunologically distinguish endogenous cofilin of mouse C3H-2K cells and recombinant porcine cofilin expressed in the same cells, a tag of an extra-nonapeptide (see Materials and Methods) was added to the N-terminus of the porcine cofilin and antibodies against the tag were used.

C3H-2K cells were co-transfected with pAcof9x, a plasmid containing porcine brain cofilin cDNA to be expressed under the human β-actin promoter and pMAM-neo containing *neo*^{r} gene. Co-transfectants were isolated in the presence of 0.6 mg/ml G418. After two cycles of single colony isolation, several transformants stably expressing recombinant pig cofilin were established for each transfection.

To examine the possibility of whether or not KKRKK, the 30 to 34th amino acid residues of cofilin, is

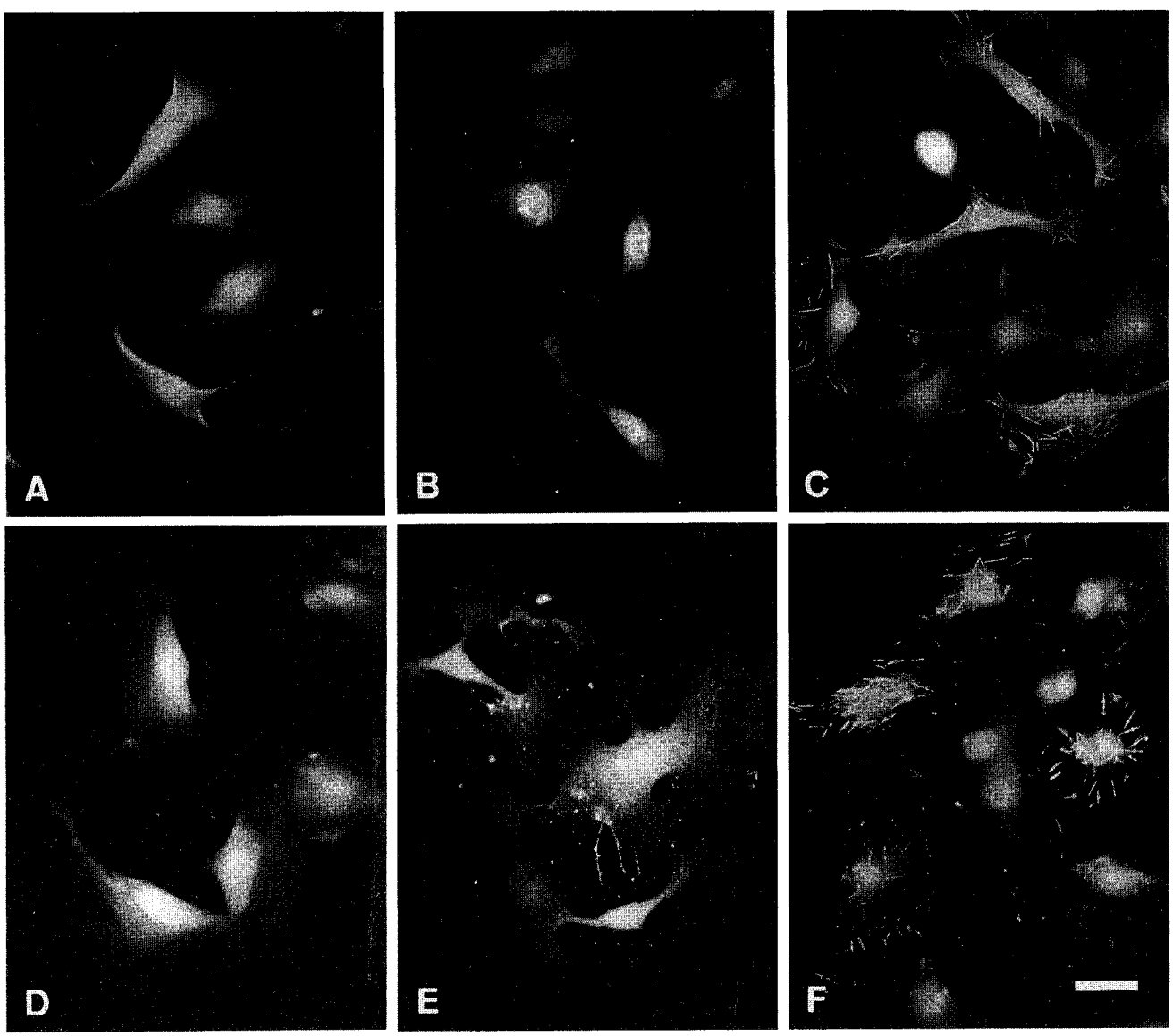

Fig. 2. Distribution of recombinant KKRKK- and KTLKK-cofilin in heat shocked cells and in cells incubated in NaCl buffer.
A2-21 (A, B, C) and A3-91 (D, E, F) cells were immunofluorescently stained with anti-peptide antibodies. (A, D), control; (B, E), incubated at 43°C for 60 min; (C, F), incubated at 37°C for 60 min in NaCl buffer. Bar = 50 μm.

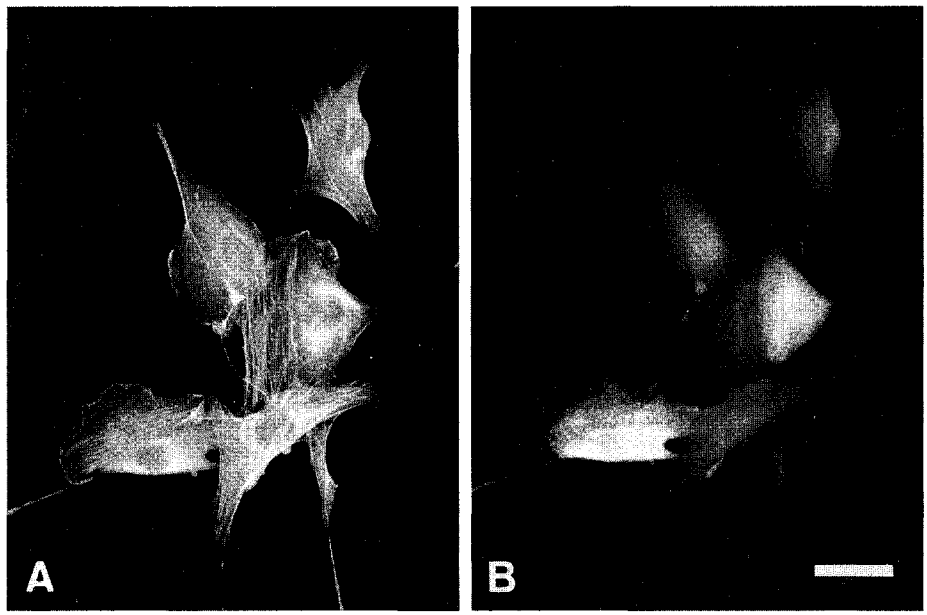

Fig. 3. Distribution of actin and recombinant cofilin in A2-21 cells.
(A), staining with FITC-anti-actin antibodies. (B), staining with anti-peptide antibodies and RH-G-anti-RIg. Bar = 50 μm.

NLS, the sequence was altered to KTLKK by the method of site-directed mutagenesis and the mutated cofilin cDNA was also expressed in C3H-2K cells as was the unaltered cofilin cDNA. The expression level of porcine cofilin in C3H-2K cells was determined by immunoblotting using both anti-peptide antibodies and anti-cofilin antibodies (Fig. 1). Expressed porcine cofilin migrated slower in SDS-polyacrylamide gel electrophoresis than endogenous mouse cofilin because the expressed cofilin contained the extra-nonapeptide at its N-terminus (Fig. 1b). The expression of transfected porcine cofilin was comparable to that of endogenous cofilin in a transfectant, A2-21 harboring plasmid DNA encoding the normal cofilin sequence. Another transfectant, A3-91 with a plasmid DNA encoding the altered sequence of porcine cofilin also expressed endogenous cofilin and the recombinant cofilin to the same extent. For control experiments, a G418-resistant transfectant, A1, was made using a plasmid containing no cofilin sequence. All of C3H-2K, A1, A2-21 and A3-91 are similar to each other in various properties including; (i) growth rate in sparse culture, (ii) cell density of confluent culture, and (iii) cell morphology.

Untreated A2-21 and A3-91 cells were separately fixed, permeabilized and labeled with the anti-peptide antibodies by indirect immunofluorescence method. The recombinant porcine cofilin was uniformly distributed in both A2-21 and A3-91 cells (Fig. 2A and 2D). The nuclear stainings were due to localization of recombinant porcine cofilin in nuclei and also to non-specific binding of anti-peptide antibodies to nuclei. The non-specific binding did not disturb the determination of the distribution of recombinant porcine cofilin, however. When A2-21 cells were incubated at 43.0°C for 60 min, the recombinant cofilin accumulated in nuclei and formed rod structures (Fig. 2B). The rods were found to contain actin (see below), as has been previously reported (8, 29). By contrast, when A3-91 cells were heated, KTLKK-cofilin did not accumulate in nuclei and no rod structures stained with anti-peptide antibodies were detected (Fig. 2E). Cytoplasmic rod formation of recombinant porcine cofilin was observed when both A2-21 and A3-91 cells were incubated at 37°C for 60 min in a NaCl buffer (7) (Fig. 2C, and 2F).

Since A3-91 cells expressed both endogenous mouse KKRKK-cofilin and porcine KTLKK-cofilin (Fig. 1), intranuclear actin rods could be formed together with the endogenous cofilin in these cells upon heat shock. To determine the distribution of both actin and the recombinant porcine cofilin, a double fluorescence staining method was applied. The recombinant porcine cofilin was visualized by the indirect immunofluorescence method as described above. Actin was visualized by direct immunofluorescence staining using FITC-labeled rabbit anti-actin antibodies in the presence of an excess amount of normal rabbit IgG. When untreated A2-21

Fig. 4. Distribution of actin and recombinant cofilin in heat shocked A2-21 and A3-91 cells.
Heat shocked A2-21 (A, B) and A3-91 (C, D) cells were doubly immunofluorescently stained with anti-actin antibodies (A, C) and with anti-peptide antibodies (B, D). Bar = 50 μ.

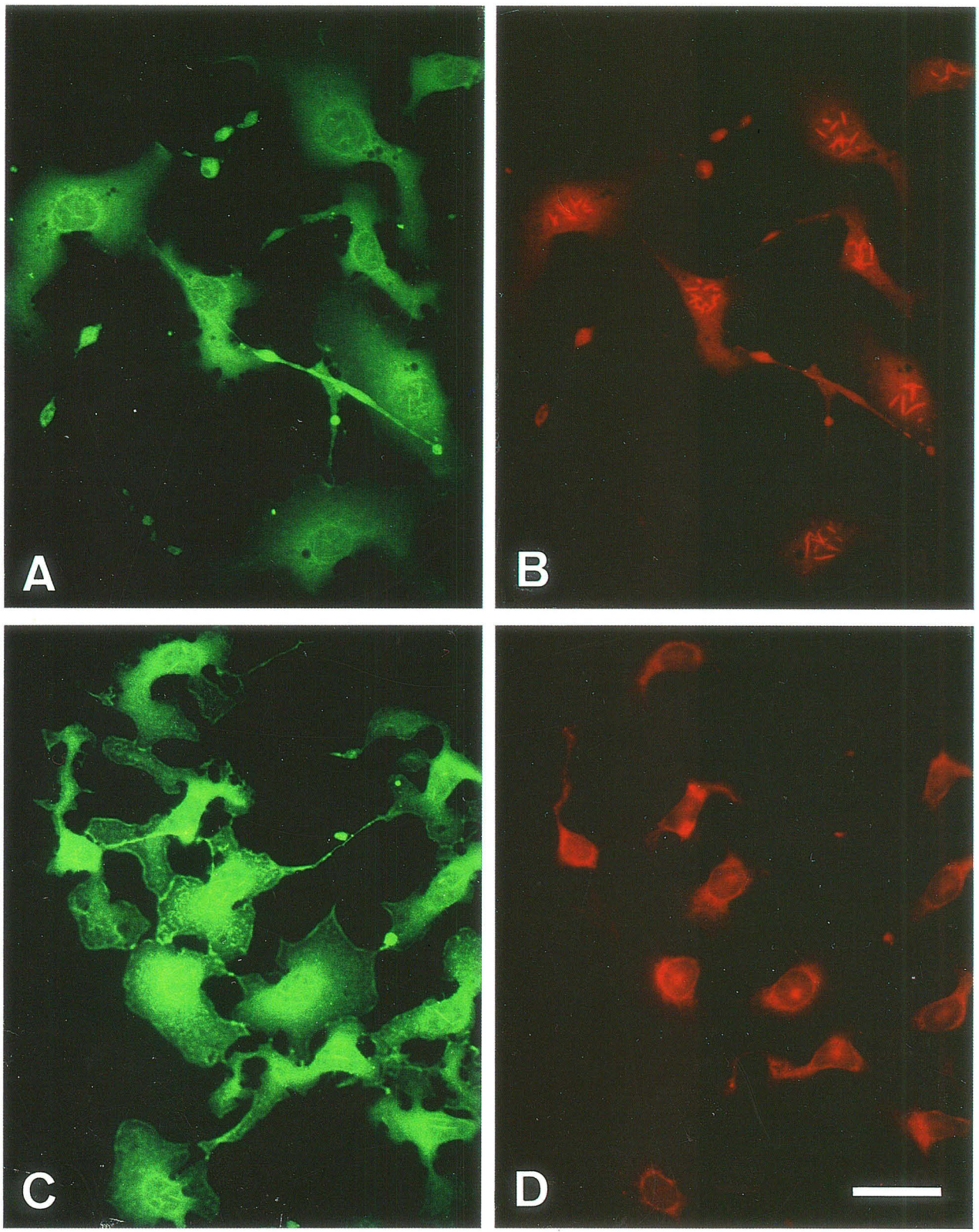

Fig. 4.

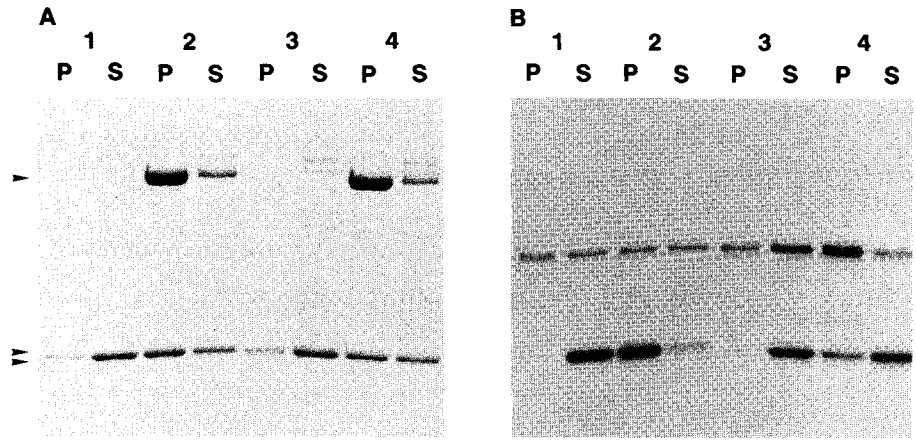

Fig. 5. Binding of KKRKK- and KTLKK-cofilin to polymerized actin.
[^{35}S]methionine-labeled recombinant KKRKK-cofilin (1 and 2) or KTLKK-cofilin (3 and 4) were mixed with unlabeled recombinant KKRKK-cofilin and were incubated with (2 and 4) or without (1 and 3) actin under actin polymerization conditions. Pellet (*p*) and supernatant (*s*) fractions were collected by centrifugation and resolved in 10–20% SDS-PAGE. A; CBB staining, B; fluorography. Single and double arrowheads indicate actin and recombinant cofilin, respectively. The radiolabeled band seen between actin and cofilin bands is a product of the ampicillin-resistant gene of pTcof9x.

cells were doubly immunofluorescently labeled, actin was found to form mainly stress fibers (Fig. 3A) and porcine cofilin was uniformly distributed in the same cells (Fig. 3B). A2-21 and A3-91 cells were incubated at 43°C and were then doubly immunofluorescently labeled. Both actin and porcine KKRKK-cofilin were translocated into nuclei and formed rod structures in heated A2-21 cells (Fig. 4A and 4B). As was expected, the results with A3-91 cells expressing KTLKK-cofilin showed that actin formed nuclear rods whereas the mutated cofilin did not (Fig. 4C and 4D).

We have compared the binding activity of KTLKK-cofilin to polymerized actin and that of KKRKK-cofilin. [^{35}S]methionine-labeled products of the *in vitro* transcription and translation system (34) for KKRKK- or KTLKK-cofilin were mixed with purified rabbit skeletal muscle actin and purified recombinant KKRKK-cofilin under actin polymerization conditions. The mixtures were centrifuged to pellet down polymerized actin together with its associated proteins. Both pellet and supernatant fractions were separately resolved in SDS-PAGE, followed by fluorography. The same amounts of unlabeled recombinant KKRKK-cofilin were coprecipitated with polymerized actin from the mixtures containing [^{35}S]methionine-labeled KKRKK- and KTLKK-cofilin (Fig. 5A). The results also indicated that a larger amount of [^{35}S]methionine-labeled KKRKK-cofilin was coprecipitated with polymerized actin than radiolabeled KTLKK-cofilin was (Fig. 5B). This suggests that the affinity of KKRKK-cofilin to actin filaments is higher than that of KTLKK-cofilin.

DISCUSSION

Proteins which exhibit their functions in nuclei are divided into two types. One includes resident proteins in nuclei which are transmitted to nuclei soon after their synthesis. For instance, histone, nucleoplasmin, and SV40 large T antigen belong to this type (3). The other includes proteins that are translocated into nuclei depending upon stimuli. Some of the transcription factors such as glucocorticoid receptors (22) and NF-κB (12) belong to this type. Nuclear location signals (NLSs) have been identified for both types. NLS appears to be much more simple for nucleus-resident proteins than for those that are stimuli-dependently translocated to nuclei. NLSs of nucleus-resident proteins may be defined as short stretches of basic amino acid residues. For instance, NLS of SV40 large T antigen has been identified as PKKKRKV in which a continuous stretch of five basic amino acid residues is critically important (9). By contrast, NLSs for those that are translocated to nuclei after receiving signals are complicated. For instance, a NLS for glucocorticoid receptor which functions after binding with glucocorticoid was mapped in separate regions of a single polypeptide which might cooperatively function upon receiving stimulatory signals.

Cofilin is a protein with molecular weight of ca. 18,500 (13) and is, therefore, expected to freely pass through nuclear pores (see ref. 3 for review) if it exists as a free monomeric form. In fact, nuclei were immunofluorescently stained with anti-cofilin antibodies to some extent (see Fig. 2A, 2D, 3B), which suggested that cofilin is present also in the nuclei of normally growing

cultured cells. Cofilin forms a 1 : 1 (mol/mol) complex with actin (16). The molecular mass of the complex is 60-kDa, and may require a NLS for translocation into nuclei. Cofilin accumulates in nuclei upon heat shock, and therefore belongs to the second type of nuclear proteins described above. A candidate for NLS of cofilin is a simple stretch of five basic amino acids, KKRKK. To examine the possibility of whether the KKRKK is a NLS of cofilin, we have employed the site-directed mutagenesis technique to alter the KKRKK sequence to KTLKK. The mutated cofilin was not induced to form intranuclear rods in its transfectants upon heat shock although endogenous cofilin formed intranuclear rods together with actin in the same cells. These results exclude the possibility that the recipient cells have lost the capability to induce heat shock-dependent translocation of actin/cofilin into nuclei and to form intranuclear actin/cofilin rods for any reason of possible physiological defects caused by transfection or/and expression of KTLKK-cofilin. Furthermore, independently isolated transfectants of C3H-2K with KTLKK-cofilin cDNA showed the same results. These results strongly suggest that the KKRKK sequence of cofilin functions as a NLS in heat shocked cells.

KTLKK-cofilin was shown to form cytoplasmic actin/cofilin rods. This fact suggests that the KTLKK-cofilin has the capability to bind polymerized actin in living cells. Determination of binding to polymerized actin for KKRKK- and KTLKK-cofilin revealed that KKRKK-cofilin has higher affinity to actin than KTLKK-cofilin. We interpreted these results as the indication that the KKRKK sequence of cofilin functions as a NLS but the alteration of KKRKK to KTLKK also indirectly affects the actin-binding ability of the molecule probably through conformational change. This interpretation is compatible with the fact that cofilin has, in its C-terminal half, partially homologous sequences to other actin-binding proteins while KKRKK is located in its N-terminal half (13). Indeed, chemical cross-linking experiments have revealed that a dodecapeptide sequence located in the C-terminal half of cofilin is directly involved in binding to actin (33).

Actin does not have any NLS-like sequence (27, 28). Since cofilin forms a 1 : 1 (mol/mol) complex with actin (16), it is possible that cofilin brings actin to the nucleus in response to heat shock. This idea is compatible with the following observations. Firstly, cofilin is generally less abundant in cultured cells than actin (17). Secondly, only the nuclei but not the cytoplasm of heat shocked cells were immunofluorescently stained with anti-cofilin antibodies whereas anti-actin antibodies stained the cytoplasm of the same cells (17). This result suggested that the amount of actin translocated into the nuclei was limited by the amount of cofilin present in heat shocked cells.

The biological significance of the translocation of actin/cofilin into the nucleus and the formation of actin/cofilin rods has to be clarified. Intranuclear rod formation may be only a consequence caused by excess amounts of actin and cofilin in the nuclei. Other than heat shock, treatment of cells with dimethyl sulfoxide (5), cytochalasin D (31), calcium ionophore (19), forskolin (21), or trifluoperazine (21) have been reported to induce the formation of intranuclear actin rods. All of these treatments cause the alteration or modulation of nuclear events. For instance, heat shock and dimethyl sulfoxide induce alterations in gene expression (4) and cytochalasins modulate RNA splicing (24). Translocation of actin/cofilin into the nuclei may cause the modulation of nuclear skeleton which provides the scaffold for such nuclear events.

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