Journal of Cellular Biochemistry 87:65–74 (2002)

C2PA Is a Nuclear Protein Implicated in the Heat Shock Response

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Abstract C2PA is a protein of unknown function that is expressed in spermatocytes. PDZ-RGS3 is a signaling molecule whose PDZ domain binds Ephrin-B2 and mediates reverse signaling of this protein. C2PA and PDZ-RGS3 have identical PDZ domains. To explore the function of C2PA, we compared it with PDZ-RGS3 with respect to tissue distribution, subcellular localization, and biochemistry. C2PA is expressed only in testis, whereas PDZ-RGS3 is expressed in various tissues including brain, heart, lung, liver, spleen, kidney, small intestine, skeletal muscles, and testis. These proteins also differ in their subcellular distribution, in that PDZ-RGS3 is cytosolic while C2PA is exclusively nuclear. C2PA is distributed diffusely in the nucleus and forms a few foci at 37°C. However, when cells are exposed to 42°C, the number of C2PA foci is increased. These heat shock-induced foci colocalize with CREB-binding protein and heat shock factor-1. In contrast, the distribution of PDZ-RGS3 does not change during heat stress. When overexpressed, C2PA induces heat shock response element (HSE)-dependent gene transcription, whereas PDZ-RGS3 does not. These data suggest that the function of C2PA is distinct from that of PDZ-RGS3, and that C2PA may be involved in the heat shock response in testis. J. Cell. Biochem. 87: 65–74, 2002. © 2002 Wiley-Liss, Inc.

Key words: C2 domain; PDZ domain; heat shock

Mouse C2PA is a protein with a modular structure composed of a C2 domain, a PDZ domain, and an ATP/GTP-binding motif [Linares et al., 2000]. In adults, significant levels of C2PA mRNA are detected only in testis, and C2PA is present in spermatocytes but not in spermatogonia or spermatids. Thereby, C2PA is considered to be involved in phase-specific signaling during spermatogenesis. Mouse PDZ-RGS3 was identified as a protein that binds to the C-terminus of Ephrin-B [Lu et al., 2001]. PDZ-RGS3 also contains a PDZ domain (constituting the Ephrin-B-binding domain), an ATP/GTP-binding motif, and a regulator of heterotrimeric G protein signaling (RGS) domain. EphB receptor binds Ephrin-B and inhibits the chemoattraction of cerebellar granule cells triggered by stromal cell-derived factor-1. Because stromal cell-derived factor-1 binds to the heterotrimeric G protein-coupled receptor CXCR4, a model has been proposed in which the binding of EphB to the Ephrin-B activates PDZ-RGS3, which then acts as a GTPase-activating protein for the G protein coupled to CXCR4 [Ma et al., 1998; Zou et al., 1998; Lu et al., 2001]. Mouse C2PA and PDZ-RGS3 have identical amino acid sequences in their central region that also contains the PDZ domain. Since Ephrin-B interacts with the PDZ domain of PDZ-RGS3, it is possible that the PDZ domain of C2PA may bind Ephrin-B and either potentiate or interfere with the reverse signaling of Ephrin-B. This supposition led us to characterize C2PA. We first compared the tissue and subcellular distribution of C2PA and PDZ-RGS3 and report that C2PA is a nuclear protein, whereas PDZ-RGS3 is cytosolic.

Abbreviations used: CBP, CREB-binding protein; HSE, heat shock response element; HSF, heat shock factor; PBS, phosphate buffered saline; PML, promyelocytic leukemia gene product; RGS, regulator of heterotrimeric G protein signaling.

Grant sponsor: Ministry of Education, Culture, Sports, Science, and Technology; Grant sponsor: NOVARTIS Foundation (Japan) for the Promotion of Science; Grant sponsor: Yamanouchi Foundation for Research on Metabolic Disorders.

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Received 18 June 2002; Accepted 25 June 2002

DOI 10.1002/jcb.10279

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C2PA does not colocalize with Ephrin-B, suggesting that C2PA is not involved in Ephrin-B-mediated signaling. We also showed that C2PA forms foci when cells are exposed to heat stress, and that C2PA is implicated in heat shock response element (HSE)-dependent gene transcription.

METHODS

Construction of Expression Vectors

cDNAs for mouse C2PA, mouse PDZ-RGS3, human Ephrin-B2, and human heat shock factor (HSF) 1 were obtained by PCR using cDNAs templates obtained from mouse lung, mouse brain, and human brain, respectively. Plasmids pGex4T-1 and pClneo were purchased from Amersham Pharmacia Biotech (Piscataway, NJ) and Promega (Madison, WI), respectively. pClneoMyc was constructed as described previously [Hirao et al., 1998]. To generate pMX YFP, PCR was performed using the forward and reverse primers agatctctagcgctaccggtcgccacc and gaattcacgcgtggggatcctgtacagctcgtccatgccgagagt, respectively, and pEYFP-C1 (Clontech, Palo Alto, CA) as template, and the PCR product was digested with Bgl II/EcoR I and ligated into BamH I/EcoR I sites of pMX puro. Expression vectors for C2PA, PDZ-RGS3, Ephrin-B2, and HSF1 were constructed by conventional molecular biology techniques and PCR methods using vectors encoding these proteins. pSRα luciferase was a generous gift of Dr. S. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo). pGex4T-1 C2PA-3 contained the C2 and PDZ domains of C2PA (amino acid residues 1-280), while pClneo C2PA and pClneoMyc C2PA contained the entire C2PA coding region. pClneoMyc PDZ-RGS3 and pClneo Ephrin-B2 contained the full-length mouse PDZ-RGS3 and human Ephrin-B2, respectively. pHSE-SEAP and pSRE-SEAP were obtained from Clontech (Palo Alto, CA).

Antibodies

Rabbit anti-C2PA was raised against the product of pGex4T-1 C2PA-3. Mouse anti-Myc-tag monoclonal antibody 9E10 was obtained from American Type Culture Collection. Mouse anti-SC-35, rabbit anti-CREB-binding protein (CBP), and rabbit anti-Ephrin-B were purchased from BD Pharmingen (San Diego, CA), Upstate Biotechnology (Lake Placid, NY), and Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), respectively. Rhodamine- and fluorescein isothiocyanate-conjugated secondary antibodies for dual labeling were purchased from Chemicon (Temecula, CA). Anti-coilin [Carmo-Fonseca et al., 1992] and the anti-SMN [Liu and Dreyfuss, 1996] were generous gifts of Dr. A.I. Lamond (University of Dundee) and Dr. G. Dreyfuss (University of Pennsylvania), respectively.

Cell Culture and Transfection

To achieve high transfection efficiencies, COS cells were used for the reporter assay. HeLa cells were used for the analysis of nuclear localization, since this cell line has been used consistently to study the structure of the nucleus. COS and HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin under 5% CO_2 at 37°C. COS cells were transfected using the DEAE dextran method for all experiments, except reporter assays for which the calcium phosphate coprecipitation method was used. HeLa cells were transfected using Transfast reagent (Promega, Madison, WI).

Immunofluorescence

COS and HeLa cells were fixed with 4% (w/v) formaldehyde in phosphate buffered saline (PBS) (2.9 mM NaH_2PO_4, 9.0 mM Na_2HPO_4, 137 mM NaCl) at room temperature for 15 min and blocked with 50 mM glycine in PBS for 30 min. Samples were then incubated with 0.2% (w/v) Triton X-100 in PBS for 15 min and subsequently with 1% (w/v) bovine serum albumin in PBS at room temperature for 30 min. Samples were incubated with various primary antibodies and visualized with rhodamine- or fluorescent isothiocyanate-conjugated secondary antibodies. Fluorescent images were obtained by confocal microscopy (Zeiss LSM 510).

Subcellular Fractionation of COS Cells

Subcellular fractionation of COS cells expressing C2PA was performed as previously described [Brott et al., 1998]. Briefly, COS cells from two confluent 10-cm plates were homogenized using a Dounce homogenizer with 25 strokes in 500 μl hypotonic buffer (5 mM Tris/HCl pH 7.4 containing 1 mM EGTA, 1 mM EDTA, 2 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM (*p*-amidinophenyl) methanesulfonyl

fluoride hydrochloride). The homogenate was layered onto 500 μl of 1.1 M sucrose in hypotonic buffer and centrifuged at 1,500*g* for 10 min at 4°C. A 100 μl aliquot was collected from the upper layer of the supernatant and designated as the cytosolic and membrane fraction. The remaining supernatant was discarded and the pellet was resuspended in 1 ml of 1.1 M sucrose in hypotonic buffer and centrifuged at 1,500*g* for 5 min at 4°C. This pellet was designated as the nuclear fraction.

Reporter Assay

Plasmids were prepared using Qiagen Plasmid Midi kit (Qiagen, Hilden, Germany). COS cells grown on 3.5-cm plates were transfected with 1 μg pHSE-SEAP or pSRE-SEAP and 5 ng pSRα luciferase in combination with other plasmids. After 48 h of culture under 5% CO_2 at 37°C, the culture medium was replaced with fresh medium. Half of the cells were further incubated for 6 h under the same conditions, while the other half were incubated at 42°C for 30 min, after which they were returned to 37°C and incubated for 5.5 h. Media were collected for the SEAP assay (SEAP reporter gene assay kit, Roche Molecular Biochemicals, Mannheim, Germany) and the cells were collected and homogenized for the luciferase assay using PicaGene (Wako Chemicals, Osaka, Japan) as a substrate.

Other Procedures

Western blotting was performed using ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Total RNA was isolated from mouse tissues using the SV total RNA isolation system (Promega, Madison, WI).

RESULTS

C2PA and PDZ-RGS3 Are Overlapping Gene Products

Mouse C2PA and PDZ-RGS3 share many structural similarities (Fig. 1A). Both proteins share a common internal domain but contain different combinations of N- and C-terminal cassettes. A human homolog of mouse C2PA (FLJ20370) is present in the GenBank database, but this sequence begins with a different methionine and lacks the C2 domain. Thereby, we performed a BLAST nucleotide homology search on the human genome using mouse C2PA and PDZ-RGS3 cDNA sequences. Both genes were located on a segment of the human chromosome 9 working draft sequence (NT017568). The amino acid sequence 1–194 of C2PA is unique and not shared by PDZ-RGS3. The first 34 residues of mouse C2PA as well as residues 177–194 could not be mapped on the current human genome, due most likely to either genetic divergence between mice and human or the fact that the human genome sequence is still somewhat incomplete. However, the remainder of the mouse C2PA coding sequence could be mapped on the human genome. Amino acid residues 35–176 are encoded by six exons (numbered C1 to C6) spanning ~10 kb (Fig. 1B). Residues 195–575 of mouse C2PA are common to mouse C2PA and PDZ-RGS3 and are encoded by eight exons (numbered C7/P2 to C14/P9) spanning ~30 kb. The last 35 residues of mouse C2PA are encoded by exon C15 and are unique to this protein. The first 17 amino acids of mouse PDZ-RGS3 are encoded by exon P1, located between exons C6 and C7/P2, and residues 399–930 are encoded by eight exons numbered P10 to P17. C15 is located between C14/P9 and P10. Human C2PA contains exons C7 to C15 but has a N-terminal exon that does not appear in mouse C2PA. The overall homology between human and mouse C2PA is 88% at the nucleotide level. During the revision of this work, human PDZ-RGS3 (GenBank accession number AF463495) and C2PA-RGS3 (GenBank accession number AF490839) were deposited. We also performed a BLAST nucleotide homology search on the human genome using human PDZ-RGS3 sequence. The intron–exon organization of human PDZ-RGS3 is similar to that of mouse PDZ-RGS3 (data not shown). Human C2PA-RGS3 is composed of different combination of exons and has C2, PDZ, and RGS domains.

Northern blot analysis of mouse C2PA mRNA was reported previously [Linares et al., 2000]. Their probe covered the PDZ domain and the ATP/GTP-binding motif, and the mRNA for PDZ-RGS3 was detected as well. We prepared a mouse C2PA-specific DNA probe corresponding to C2PA amino acid residues 1–156 and a mouse PDZ-RGS3-specific probe corresponding to PDZ-RGS3 residues 504–930 and performed Northern analyses using adult mouse tissues. Two C2PA mRNA bands of ~5.2 and ~3.1 kb were detected in the testis only (Fig. 2A). In contrast, PDZ-RGS3 mRNA was expressed

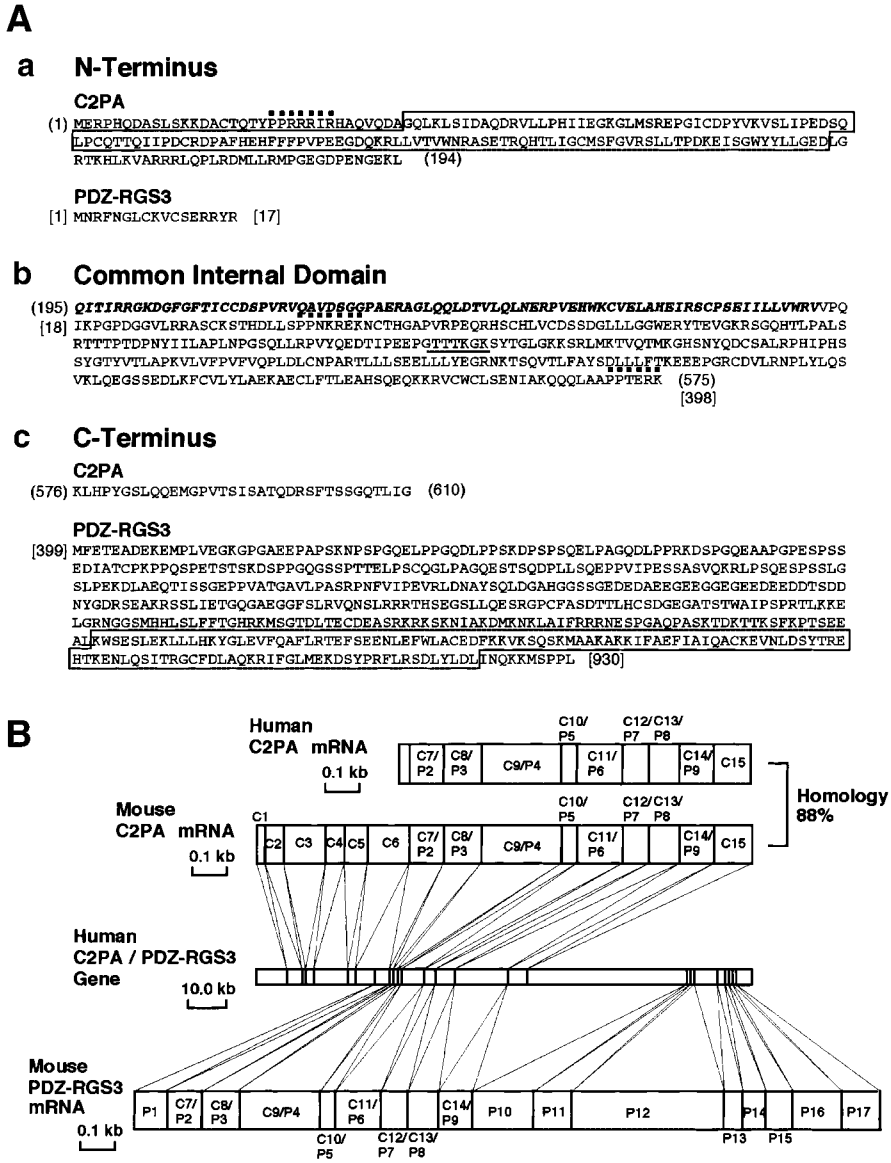

Fig. 1. Alignment of the amino acid sequences of mouse C2PA and mouse PDZ-RGS3 and intron–exon organization of the human C2PA/PDZ-RGS3 gene. **A**: Sequences of mouse C2PA and mouse PDZ-RGS3. The numbers in parentheses and in brackets indicate the numbers of amino acid residues of mouse C2PA and mouse PDZ-RGS3, respectively. The C2 domain and the RGS domain are boxed. The PDZ domain is shown by italic letters. The ATP/GTP-binding motif is underlined. The putative nuclear localization signals are indicated with dots; (**a**) N-terminal regions of mouse C2PA and mouse PDZ-RGS3. The amino acid residues 1–194 of mouse C2PA and 1–17 of mouse PDZ-RGS3 are specific for mouse C2PA and mouse PDZ-RGS3, (**b**) internal regions common to mouse C2PA and mouse PDZ-RGS3. The amino acid residues 195–575 of mouse C2PA and 18–398 of mouse PDZ-RGS3 are identical, (**c**) C-terminal regions of mouse C2PA and mouse PDZ-RGS3. The amino acid residues 576–610 of mouse C2PA and 399–930 of mouse PDZ-RGS3 are specific for mouse C2PA and mouse PDZ-RGS3. **B**: Intron–exon organization of the human C2PA/PDZ-RGS3 gene. Mouse C2PA mRNA and mouse PDZ-RGS3 mRNA are shown above and below human C2PA/PDZ-RGS3 gene. Human C2PA mRNA is indicated at the top. The homology between human and mouse C2PA is 88%. The positions of the exons of human C2PA/PDZ-RGS3 gene are shown as the lines. C1 to C6 and C15 are exons specific for mouse C2PA. P1 and P10 to P17 are exons specific for mouse PDZ-RGS3. C7/P2 to C14/P9 are common for mouse C2PA and mouse PDZ-RGS3. kb, kilobases.

in brain, heart, lung, liver, spleen, kidney, small intestine, skeletal muscles, and testis (Fig. 2B); and all these tissues showed bands of ~4.0 and ~2.7 kb. All lanes showed similar signals for control β-actin (data not shown). Thus, the distribution of C2PA and PDZ-RGS3 mRNAs in adult mouse tissues is quite different.

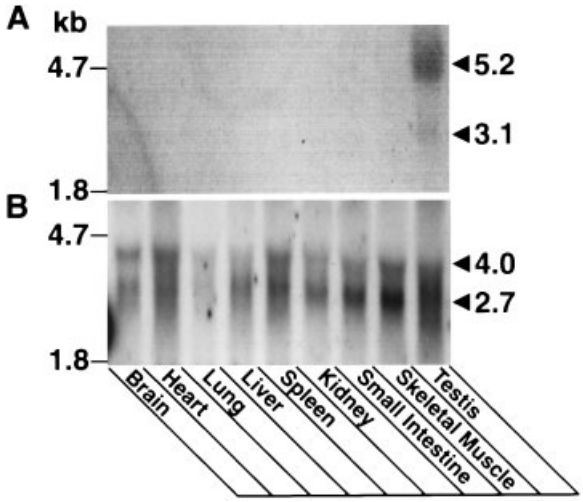

Fig. 2. Northern blot analyses of mouse C2PA and mouse PDZ-RGS3. Uniformly labeled DNA probes corresponding to the amino acids 1–156 of mouse C2PA and 504–930 of mouse PDZ-RGS3 were prepared. Blots with 20 μg of total RNA from each mouse tissue were hybridized with probes (3,600,000 cpm/each probe) and exposed for 8 days. **A**: Northern blot of mouse C2PA. **B**: Northern blot of mouse PDZ-RGS3. Numbers on the left indicate the size of markers. Arrow heads on the right indicate the positions of signals. kb, kilobases.

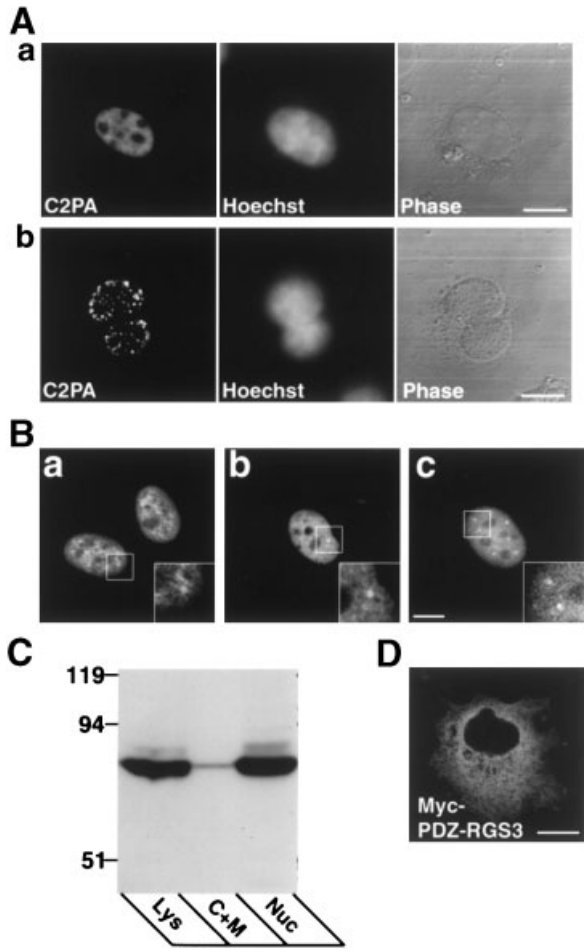

Fig. 3. Subcellular localization of C2PA and PDZ-RGS3. **A**: C2PA in COS cells. COS cells were transfected with pClneo C2PA and double-stained with the anti-C2PA antibody and Hoechst 33342. C2PA, the staining with the anti-C2PA antibody; Hoechst, the staining with Hoechst 33342; and Phase, the image of phase contrast; (**a**) diffuse staining of C2PA in the nucleus. About 76% cells show the diffuse distribution of C2PA in the nucleus, (**b**) focal staining of C2PA in the nucleus. C2PA form foci in the nucleus in 24% of cells. Bars, 10 μm. **B**: C2PA in HeLa cells. HeLa cells were transfected with pClneo C2PA, pClneoMyc C2PA, or pMX YFP C2PA. C2PA was distributed diffusely in the nucleus with foci. The insets show the demarcated areas at a higher magnification; (**a**) C2PA, (**b**) Myc-C2PA, (**c**) YFP-C2PA. Bar, 10 μm. **C**: Subcellular fractionation of COS cells expressing Myc-C2PA. The comparable amount of each fraction was immunoblotted with the anti-Myc antibody. Lys, the total lysate before the fractionation; C + M, the cytosolic and membrane fraction; and Nuc, the nuclear fraction. **D**: Myc-PDZ-RGS3 in COS cells. COS cells were transfected with pClneoMyc PDZ-RGS3 and stained with the anti-Myc antibody. Bar, 10 μm.

Nuclear Localization of C2PA

We next compared the subcellular distribution of both C2PA and PDZ-RGS3. When expressed in COS cells, C2PA exhibited diffuse localization throughout the nucleoplasm, but was excluded from nucleoli in 76% of the cells (Fig. 3Aa). In 24% of the cells, C2PA was found in foci within the nucleus (Fig. 3Ab). We confirmed that C2PA is localized in the HeLa cell nucleus (Fig. 3B), although both diffuse nuclear distribution and focus formation were observed in single cells (Fig. 3B, insets). C2PA recombinant proteins containing either Myc or YFP tags at the N-terminus were also localized to the nucleus, indicating that such fusions do not affect C2PA subcellular localization. Either Myc- or YFP-tagged C2PA was used in subsequent experiments. When HeLa cells were fractionated, Myc-C2PA was recovered in the nuclear fraction (Fig. 3C). In contrast, Myc-PDZ-RGS3 localized to the cytoplasm (Fig. 3D). Further, when coexpressed, Myc-PDZ-RGS3 and Ephrin-B2 colocalized in the cytoplasm (Fig. 4A). However, C2PA remained in the nucleus even when coexpressed with Ephrin-B2 (Fig. 4B). Despite the fact that C2PA and PDZ-RGS3 have identical PDZ domains, the above results suggest that these proteins differ in their subcellular distribution and that C2PA does not interact with Ephrin-B2 in cells.

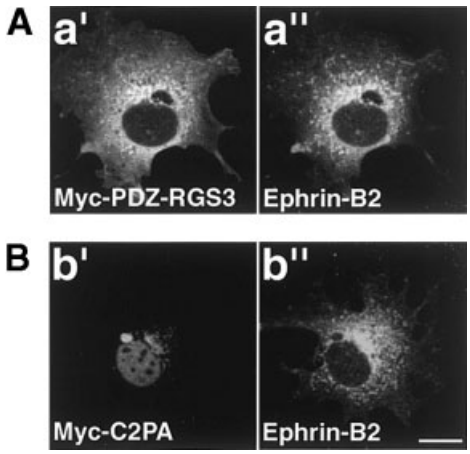

Fig. 4. Localization of PDZ-RGS3 and C2PA with Ephrin-B2 in COS cells. COS cells were transfected with pClneo Ephrin-B2 with either pClneoMyc PDZ-RGS3 or pClneoMyc C2PA and double-stained with the anti-Ephrin and the anti-Myc antibodies. **A**: Myc-PDZ-RGS3 coexpressed with Ephrin-B2 in a COS cell; (**a**′) Myc-PDZ-RGS3, (**a**″) Ephrin-B2. **B**: Myc-C2PA coexpressed with Ephrin-B2 in a COS cell; (**b**′) Myc-C2PA, (**b**″) Ephrin-B2. Bar, 10 μm.

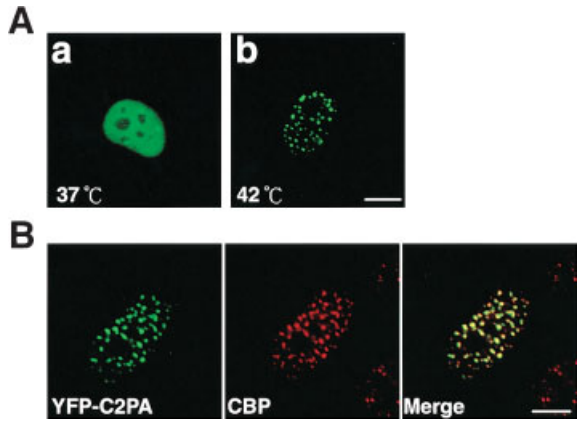

Fig. 5. Formation of C2PA foci induced by the heat stress. **A**: YFP-C2PA in HeLa cells before and after the heat stress. HeLa cells were transfected with pMX YFP C2PA and were fixed before or after the exposure to 42°C for 30 min; (**a**) before the heat stress, (**b**) after the heat stress. Bar, 20 μm. **B**: Colocalization of CBP with C2PA in HeLa cells after the heat stress. HeLa cells expressing YFP-C2PA were fixed and stained with the anti-CBP antibody after the exposure to 42°C for 30 min. Bar, 10 μm.

Effect of Heat Shock on the Nuclear Localization of C2PA

As described above, C2PA localizes to the nucleus and exhibits both diffuse and focal staining. One hypothesis to explain this dual staining pattern is that specific nuclear localization of C2PA may be governed by the cell cycle. However, we found no significant correlation between either of these types of localization and cell division in HeLa or COS cells (data not shown). The observed nuclear foci were reminiscent of speckles and coiled bodies in which snRNP proteins and splicing factors accumulate [Lamond and Earnshaw, 1998; Lewis and Tollervey, 2000]. Previous studies report that actinomycin D or amanitin treatment changes the distribution of some components of speckles and coiled bodies [Carmo-Fonseca et al., 1992]. However, neither 50 μg/ml amanitin nor 10 μg/ml actinomycin D affected either the induction or inhibition of focus formation of Myc-C2PA in HeLa cells (data not shown). We next tested the effect of heat stress on focus formation given that C2PA is expressed in spermatocytes in testis and that heat stress impairs spermatogenesis [Crew, 1922; Moore, 1951]. In HeLa cells expressing YFP-C2PA, heat stress (42°C) dramatically induced the formation of foci (Fig. 5A). We confirmed this result in similar experiments in HeLa cells expressing Myc-C2PA and in COS cells expressing C2PA (data not shown). We next examined whether C2PA in heat stress-induced foci colocalizes with markers of various subnuclear compartments [Lamond and Earnshaw, 1998; Lewis and Tollervey, 2000]. Coilin, SC-35, and the survival of motor neurons protein were used as markers for coiled bodies, speckles, and gems, respectively. In HeLa cells exposed to 42°C, YFP-C2PA did not colocalize with any of these three marker proteins (data not shown). We next examined whether C2PA colocalizes with CBP, a marker for a promyelocytic leukemia gene product (PML)-containing nuclear body [LaMorte et al., 1998]. CBP foci colocalized with YFP-C2PA in heat stressed HeLa and COS cells (Fig. 5B; data not shown). We also examined the temporal profile of C2PA/CBP foci formation in HeLa cells transiently expressing YFP-C2PA (Fig. 6A). The majority of YFP-C2PA distributed diffusely in the nucleus at 37°C, although some foci were also observed (Fig. 6A, 0 min). In cells exposed to 42°C, the number of YFP-C2PA foci increased (Fig. 6Ba). At 15 min, almost all YFP-C2PA accumulated in foci, and these foci were maintained when cells were kept at 42°C for 30 min. When cells were returned to 37°C after 30 min of heat stress, the foci disappeared and the majority of YFP-C2PA distributed diffusely in the nucleus after 30 min (Fig. 6A, 30 min after the recovery at 37°C). CBP

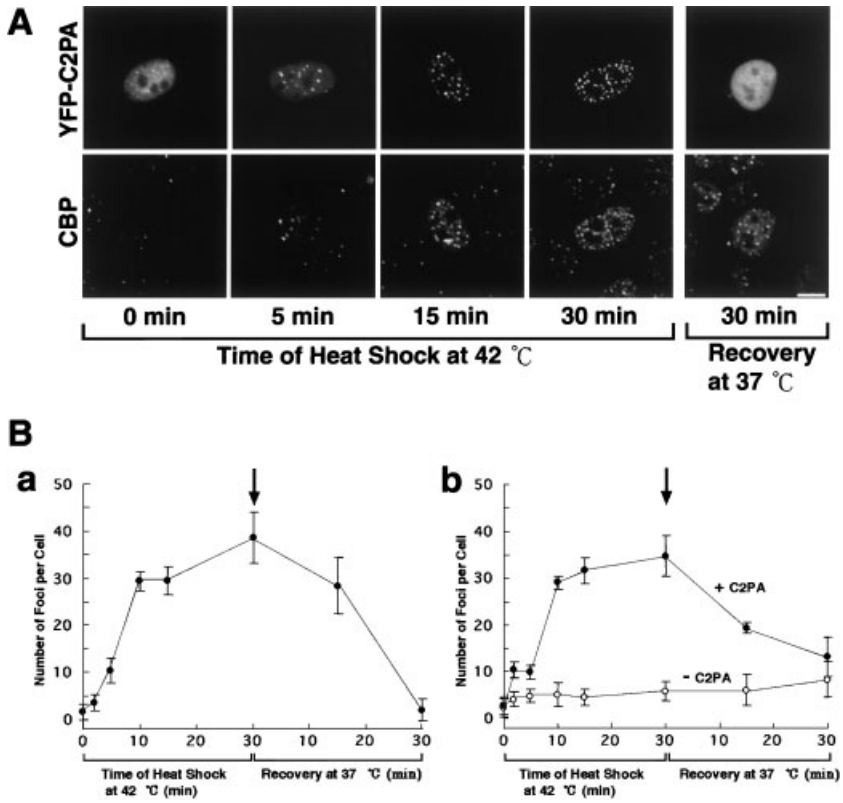

Fig. 6. Time course of the formation of C2PA foci and CBP foci in HeLa cells after the heat stress. **A**: YFP-C2PA and CBP in HeLa cells at various time points after the heat stress. HeLa cells were transfected with pMX YFP C2PA. Cells were exposed to 42°C for the indicated time, fixed, and stained with the anti-CBP antibody. After the heat stress for 30 min, cells were returned to 37°C, incubated for 30 min, fixed, and stained with the anti-CBP antibody. Bar, 10 μm. **B**: Numbers of C2PA and CBP foci at various time points after the heat stress. Numbers of C2PA and CBP foci were counted at various time points after the heat stress. The data were shown as an average of five cells with standard errors of mean. The arrows indicate the time point where cells were returned to 37°C; (**a**) the numbers of C2PA foci, (**b**) the numbers of CBP foci. Closed and open circles indicate the numbers of CBP foci in cells with and without C2PA, respectively.

appeared in foci in HeLa cells at 37°C (Fig. 6A), and the number of CBP foci increased in HeLa cells expressing YFP-C2PA during heat stress at 42°C (Fig. 6A,Bb). After 15 min of heat stress, CBP formed numerous foci that colocalized with C2PA foci. When cells were returned to 37°C, CBP foci disappeared in parallel with C2PA foci. We next compared the localization of C2PA and HSF1, a major heat shock factor. Myc-HSF1 localized exclusively to the nucleus when overexpressed in COS cells (data not shown). Under heat stress, Myc-HSF1 localized to foci that partially colocalized with C2PA (Fig. 7). These findings indicate that C2PA, together with CBP and HSF1, is recruited to the machinery involved in HSE-dependent gene transcription.

C2PA Induces HSE-Dependent Gene Transcription

To further explore the function of C2PA in HSE-dependent gene transcription, we performed reporter assays using pHSE-SEAP as the reporter and pSRα luciferase as the reference. Overexpression of Myc-C2PA induced HSE-dependent gene transcription at 37°C but did not enhance it under conditions of heat stress (Fig. 8A). In contrast, when HSF1 was coexpressed with Myc-C2PA, HSE-dependent gene transcription was enhanced under heat stress (Fig. 8B). However, Myc-PDZ-RGS3 had no effect on HSE-dependent gene transcription at 37°C or under heat stress. Finally, Myc-C2PA had no effect on serum response element-dependent gene transcription (data not shown).

DISCUSSION

The BLAST search on the human genome database suggests that C2PA and PDZ-RGS3 are overlapping genes [Linares et al., 2000; Lu et al., 2001]. However, the tissue and subcellular

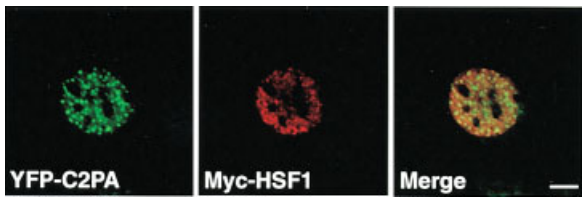

Fig. 7. Colocalization of C2PA and HSF1 in COS cells under the heat stress. COS cells were transfected with pMX YFP C2PA and pClneoMyc HSF1 and stained with the anti-Myc antibody after the exposure to 42°C for 30 min. Bar, 10 μm.

distribution of C2PA is different from that of PDZ-RGS3. C2PA is present only in testis, whereas PDZ-RGS3 is expressed ubiquitously, implying that the expression of the C2PA and PDZ-RGS3 genes is regulated in a tissue-dependent manner. Previously, Linares et al. [2000] detected one C2PA mRNA of ~4.0 kb in mice, but we detected two C2PA mRNAs of ~5.2 and ~3.1 kb in our Northern analysis. While the reason for this discrepancy is not clear, we speculate that the probe used by Linares and coworkers may have detected the 4.0 kb message of PDZ-RGS3, given that we detected two PDZ-RGS3 mRNAs of ~4.0 and ~2.7 kb. It is also unclear as to why both C2PA and PDZ-RGS3 express two mRNAs of different size, although they may be attributable to differential polyadenylation or alternative splicing. From sequences deposited in GenBank, the estimated sizes of the *C2PA* and *PDZ-RGS3* genes are 4.7 kb (mouse C2PA, AJ250999), 2.1 kb (human C2PA, FLJ20370), and 2.9 kb (mouse PDZ-RGS3, AF350047). Since our antibody to C2PA was not sensitive enough to detect the endogenous protein, at present, we are unable to determine the subcellular localization of C2PA in vivo. However, when expressed in various cells, C2PA is localized exclusively to the nucleus, whereas PDZ-RGS3 is distributed in the cytosol. C2PA has three putative nuclear localizing signals, Pro-Pro-Arg-Arg-Arg-Ile-Arg, Pro-Pro-Asn-Lys-Arg-Glu-Lys, and Pro-Pro-Thr-Glu-Arg-Lys (Fig. 1A). The latter two signals are also present in PDZ-RGS3. Therefore, the first signal may function as a nuclear localizing signal for C2PA in vivo. Alternatively, the PDZ-RGS3 RGS domain may mask this protein's nuclear localizing signal(s) thus preventing PDZ-RGS3 from entering the nucleus. RGS proteins constitute a large family [Berman and Gilman, 1998; Zheng et al., 1999], and nuclear localization was reported for one RGS member, RGS12 [Chatterjee and Fisher, 2000]. Twelve distinct transcripts are derived from the 16 exons of the RGS12 gene, and some of these gene products are localized to the nucleus.

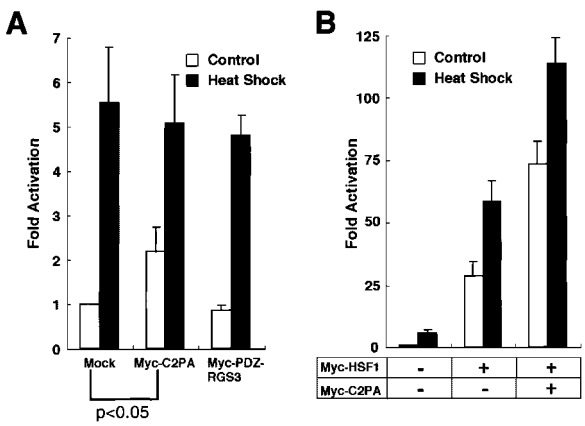

Fig. 8. Effect of C2PA on the heat shock response element (HSE)-dependent gene transcription. **A**: COS cells of 3.5 cm-plate were transfected with 1 μg pHSE-SEAP and 5 ng pSRα luciferase with the mock, pClneoMyc C2PA, or pClneoMyc PDZ-RGS3. Fourty-eight hours after the transfection, the medium was replaced. Half groups of cells were incubated at 37°C for 6 h. The other half groups were exposed to 42°C for 30 min, returned to 37°C, and incubated for 5½ h. The SEAP activities were calibrated with the luciferase activities. The data are shown as the fold activation compared with the value for COS cells transfected with 1 μg pHSE-SEAP, 5 ng pSRα luciferase, and the mock without the heat shock. All data represent the mean of three independent experiments with the standard errors of mean. There is a statistically significant difference between the values for mock-transfected and C2PA-transfected cells without the heat shock. White bars, without the heat shock; and black bars, with the heat shock. **B**: COS cells of 3.5 cm-plate were transfected with 1 μg pHSE-SEAP and 5 ng pSRα luciferase with the mock, pClneoMyc HSF1, or pClneoMyc HSF1 + pClneoMyc C2PA. The assays were performed in the same manner as described for (A). White bars, without the heat shock; and black bars, with the heat shock.

In our present work, we showed that C2PA is involved in HSE-dependent gene transcription. C2PA shows two distinct patterns of distribution within the nucleus. The majority is distributed diffusely in the nucleoplasm, although several foci are also observed. A previous study showed that the nuclear protein RGS12 localizes to foci, and that foci formation is cell cycle-dependent [Chatterjee and Fisher, 2000]. However, with regard to C2PA, we detected no difference in the formation of foci in dividing or non-dividing HeLa or COS cells. We also found that heat stress dramatically induces foci formation in that almost all C2PA is accumulated in foci 30 min after the onset of heat stress.

Recent studies revealed that there are several subnuclear compartments with distinct functions, including speckles, coiled bodies, gems, and PML-containing nuclear bodies [Lamond and Earnshaw, 1998; Lewis and Tollervey, 2000]. We tested whether C2PA foci colocalize with marker proteins for these compartments, and found that C2PA foci colocalize with CBP and HSF1. CBP is known to localize to a transcriptionally active domain in the nucleus [von Mikecz et al., 2000]. Therefore, C2PA is presumably concentrated in a compartment where gene transcription is active under heat stress. When C2PA is overexpressed, HSE-dependent gene transcription is enhanced at 37°C. We hypothesized that the foci formed during C2PA overexpression at 37°C may function as sites for active HSE-dependent gene transcription. Thus, C2PA should further enhance the HSE-dependent gene transcription after heat stress, since the number of CBP foci is larger in the presence of C2PA than in its absence. However, as shown in Figure 8A, C2PA does not change HSE-dependent gene transcription following heat stress. This discrepancy may be due to the limitation of endogenous HSF1, since HSE-dependent gene transcription following heat stress is stimulated by C2PA when HSF1 is overexpressed. The molecular mechanism by which heat stress induces changes in C2PA nuclear localization and facilitates CBP foci formation when C2PA is overexpressed remains to be clarified. Heat stress may trigger a conformational change in C2PA such that C2PA then forms foci with CBP through either direct or indirect interaction. The complex containing C2PA and CBP presumably mediates the interaction between HSF1 and the general transcriptional machinery. However, we were unable to confirm the interaction of C2PA with either CBP or HSF1, and therefore further studies must be undertaken to demonstrate that such interactions exist.

Heat stress is deleterious to spermatogenesis [Crew, 1922; Moore, 1951]. The expression of active HSF1 also arrests spermatogenesis with consequent spermatocyte apoptosis [Nakai et al., 2000]. The role of C2PA in the testes under physiological conditions or heat stress remains unknown. C2PA may bolster the heat shock response in spermatocytes, thus rendering these cells sensitive to heat stress. Recent studies revealed that heat shock factors play roles in normal development and cell proliferation [Morimoto, 1998; Pirkkala et al., 2001]. Likewise, C2PA may also amplify the function of heat shock factors involved in testis development and spermatogenesis.

ACKNOWLEDGMENTS

This study was supported by grants-in-aid for Scientific Research and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology, a grant from NOVARTIS Foundation (Japan) for the Promotion of Science (1999), and a grant from Yamanouchi Foundation for Research on Metabolic Disorders (2000). We thank Dr. A.I. Lamond (University of Dundee) for the antibody to coilin, Dr. G. Dreyfuss (John Hopkins University) for the antibody to SMN, Dr. T. Kitamura (Institute of Medical Science, University of Tokyo) for pMX puro, and Dr. S. Kato (Institute of Molecular and Cellular Biosciences, University of Tokyo) for pSRα luciferase. We also thank Ms. C. Rokukawa and Ms. M. Miyahara-Tenkatsu for skillful technical assistance.

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