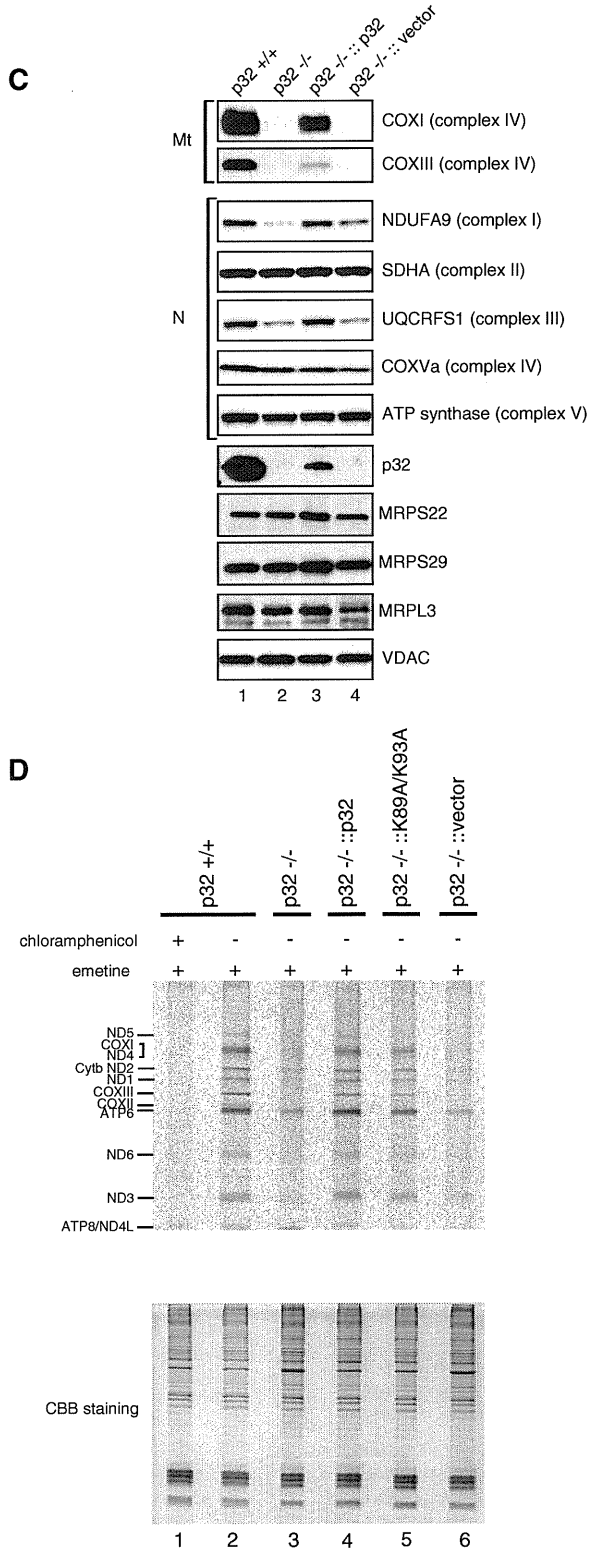

Figure 3. Continued.

respiratory chain by western blotting. Knockout of p32 resulted in a decrease in COXI and COXIII, subunit I and III of complex IV, which is encoded by mtDNA (Figure 3C). The loss of nuclear-encoded complex III subunit (UQCRFS1) may reflect the instability of the complex, which arises from general loss of mtDNA-encoded components, as shown later (Figure 3D). NDUFA9 (complex I) was slightly decreased in knockout cells. ATP synthase (complex V) barely changed. Re-expression of p32 in p32-knockout MEFs ($p32^{-/-}$::*p32*) restored mtDNA-encoded COXI, COXIII and nuclear-encoded NDUFA9, and UQCRFS1, but introduction of the vector only ($p32^{-/-}$::vector) did not rescue the levels of these proteins. There were no differences in the expression level of the complex II 70-kDa subunit (SDHA) or in the levels of mitochondrial proteins VDAC, mitochondrial ribosomal protein (MRP) S29 and S22, again indicating that p32 knockout specifically affects the levels of complexes I, III and IV.

Reduced mitochondrial translation rate

Next, to investigate the defect in mitochondrial protein synthesis, wild-type and knockout MEFs were pulse-labeled with a mixture of [^{35}S]-methionine and [^{35}S]-cysteine. To distinguish between proteins synthesized in the cytoplasm and mitochondria, cells were pre-treated with emetine and/or chloramphenicol, which are specific inhibitors of cytoplasmic and mitochondrial protein synthesis, respectively. Electrophoresis of whole-protein extracts from emetine-treated cells showed radioactive bands in the control (Figure 3D, lane 2). This profile clearly showed the products of mitochondrial protein synthesis. The synthesis of these proteins was completely inhibited by chloramphenicol (lane 1), confirming mitochondrial protein synthesis. This mitochondrial protein synthesis was strikingly reduced in p32-deficient cells (lane 3). This reduced protein synthesis was not caused by a reduction of mitochondrial mass, because there were no differences in the levels of mitochondrial proteins VDAC, MRPS29 and MRPL3, as shown in Figure 3C, which indicated that the mitochondrial mass in p32-deficient cells was normal. Re-expression of p32 in p32-deficient MEFs partially recovered the translation (lane 4). These results suggest that p32 deficiency results in a defect of mitochondrial protein synthesis.

Depletion of p32 affects mitoribosome distribution

Protein synthesis within mitochondria is performed by 55S mitoribosomes, which consist of a small 28S subunit and a large 39S subunit. There were no differences in the levels of mitoribosomal proteins such as MRPS29, MRPS22 and MRPL3, indicating that the total quantity of mitoribosomal subunits in p32-deficient cells was not largely affected (Figure 3C). To clarify the role of p32 in mitochondrial translation, we analyzed sedimentation profiles in a sucrose density gradient (Figure 4A and Supplementary Figure S4). The small and large subunits were traced with antibodies against MRPS29 and MRPL3, respectively, and were compared between wild-type, p32-knockout and p32-re-expressed MEFs. Similar levels of the 28S small subunit (fractions 5–6) and 39S large subunit (fractions 7–8) were found in the MEFs of these three cell lines. The 55S mitoribosome was mainly distributed in fractions 9 and 10 observed as the second peaks in MRPS29 and MRPL3 plots (Figure 4B).

However, in $p32^{-/-}$ cells, 55S formation were decreased. In p32-re-expressed cells, 55S ribosome formation was slightly recovered (fractions 9–10). The mitoribosomes broadly spanning the heavier fractions (fractions 11–16) may have represented translating multi-mitoribosomes or aggregated form. After treatment with EDTA in wild-type p32 MEF cells, 55S mitoribosome and heavier ribosome (fractions 11–16) were decreased and resemble as the $p32^{-/-}$ cells pattern (Supplementary Figure S4). These results suggested that p32 deficiency barely affected mitoribosome biogenesis (monosome formation), but did affect the formation of functioning 55S mitoribosomes. Though p32 mainly existed in the lighter fractions 1–4, it was also found in fractions 5–16. Considering that p32 is an abundant and multi-associated protein, p32 may functionally interact with the mitoribosome, although it is not integrated into it. Taken together, we theorize that p32 depletion somehow affects the formation of a functioning mitoribosome.

p32 binds to mitochondrial mRNA

Protein–RNA interactions play essential roles in post-transcriptional control of gene expression, including in mRNA degradation and translational regulation. p32 was originally copurified with the pre-mRNA splicing factor SRSF1 from human HeLa cells (22). In addition, p32 was designated a mitochondrial RNA-binding protein by proteomic studies (18). These studies suggest an RNA-binding ability of p32. To test whether p32 was able to bind mitochondrial mRNA, hemagglutinin-tagged p32 (p32-HA) protein was expressed in HeLa cells and immunoprecipitated from total cell lysates. RNA was extracted from the immunoprecipitate (pellet). The p32-bound mRNAs were directly quantified by qRT–PCR and northern blotting (Figure 5 and Supplementary Figure S5). After induction of p32-HA, a substantial enrichment of the immunoprecipitate was observed for all 11 mitochondrial transcripts analyzed, but cytoplasmic mRNAs (*MAPK6* and *β-actin*) were not enriched (Figure 5A, left panel), confirming that p32 was able to interact with mitochondrial mRNA *in vivo*. After IP using the anti-p32 antibody and $p32^{+/+}$ MEFs, we also observed that mitochondrial mRNAs, including ND1 and ND3 mRNAs, were enriched (Figure 5A and B), suggesting that p32 was also bound to mitochondrial mRNA in mouse cells. Northern blotting showed that ND1 and

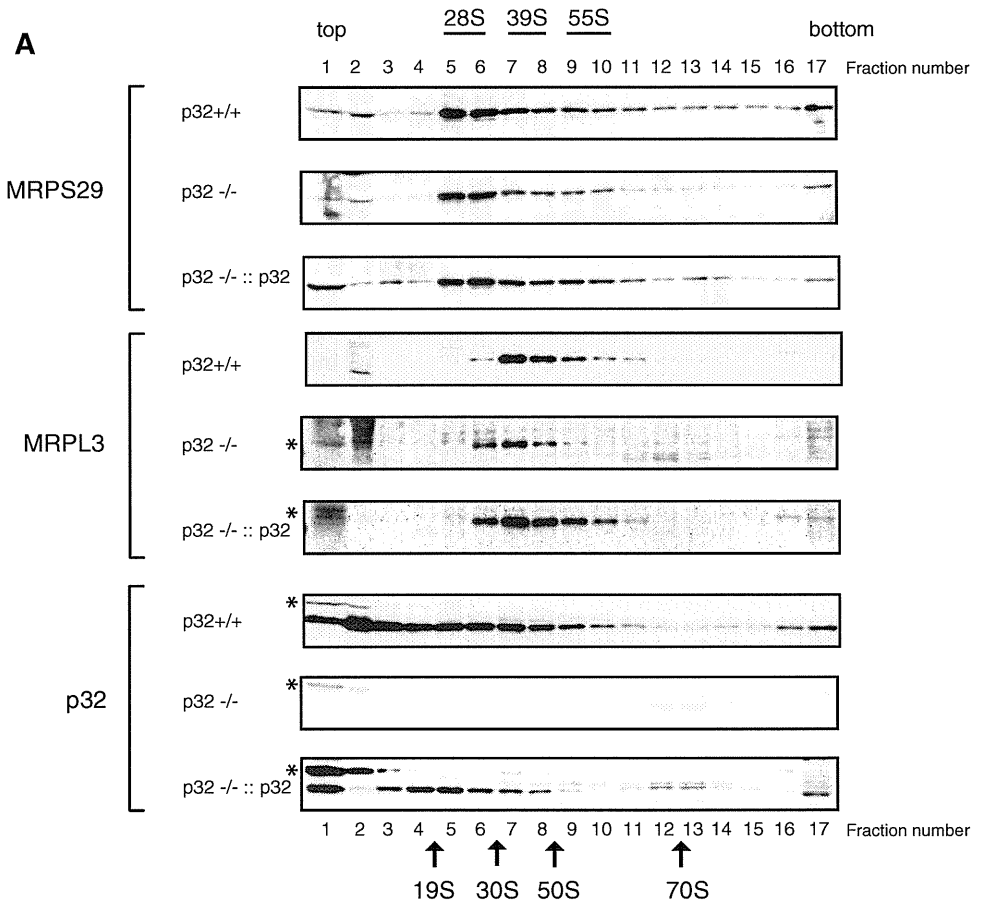

Figure 4. Reduction of p32 results in decreased mitoribosomes. (**A**) Upper panels: Sedimentation analysis of mitoribosomal particles by centrifugation via a linear 15–30% sucrose density gradient. Fraction numbers are indicated. The migration of mitoribosomal particles in wild-type (+/+), p32-knockout ($^{-/-}$) MEFs and p32 re-expressed ($^{-/-}$::p32) MEFs was determined by immunoblotting with antibodies against MRPL3 (39S large subunit), MRPS29 (28S small subunit) and p32. Arrows indicate peaks of optical density for the S value markers. Asterisk (*) shows non-specific band of MRPL3 and p32 at top fraction (1–2). (**B**) Representative blots were analyzed densitometrically. The signal intensity of the protein in each fraction was plotted. The maximum value was 100% for each protein level.

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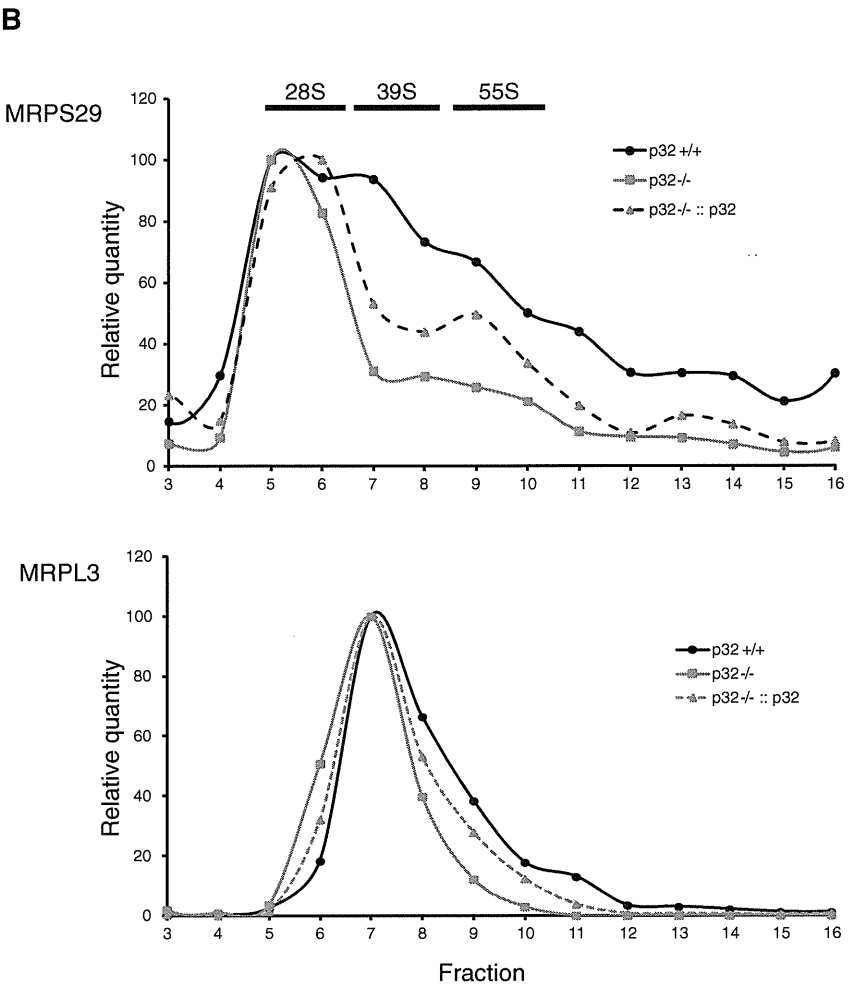

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COX1 mRNAs were detected at the same size after IP of p32, but cytoplasmic *β-actin* mRNA were not detected, which suggests that p32 was bound to these intact mitochondrial mRNAs (Figure 5C). We observed that the LRPPRC which RNA binding to mtRNA were reported (45), was not bound to mitochondrial mRNA in this experimental condition (Figure 5A, right panel) or TFAM which bind to mtDNA was also not bound (data not shown). Enrichment of *12S* and *16S* rRNAs was also observed (Figure 5A). There are no mtDNA in IP sample, because of no detection of PCR product without reverse transcriptase treatment (data not shown). 12S and 16S rRNAs were likely to be enriched via ribosome binding because western blotting of the eluates from the IPs showed that large and small mitoribosomal subunit proteins, MRPS22 and MRPL3, were co-immunoprecipitated with p32-HA (Figure 7). Taken together, these data are consistent with p32-association with all mitochondrial mRNAs in the mitochondrial matrix.

p32 binds to RNA oligonucleotides *in vitro*

Because p32 deficiency did not reduce mtDNA transcripts or affect biogenesis of mitoribosomes (Figures 3C and 4A), and p32 interacted with all mitochondrial mRNAs (Figure 5A and Figure 5B), we considered that p32 may bind to mRNAs via their poly(A) tail or in a sequence-independent manner. To test this hypothesis, we investigated the interaction of recombinant p32 protein with several RNA oligonucleotides *in vitro*. A REMSA showed that recombinant p32 protein clearly bound to a 22-mer poly(A) RNA in a dose-dependent manner (Figure 6A, lanes 1–7), while control glutathione-*S*-transferase (GST) protein and recombinant histidine (His)-tagged TFAM protein did not, indicating that this interaction was not mediated by the GST- or His-tag (Figure 6A, lanes 14-19). An interaction was not observed with a single-stranded, 22-mer poly(A) DNA oligonucleotide (Figure 6B, lanes 13–15), indicating that the p32 interaction was RNA-specific. The interaction with the 22-mer poly(U) RNA oligonucleotide was very weak (lanes 7–9). We also observed that recombinant p32 protein clearly bound to random 14- and 40-mer RNAs in a dose-dependent manner (Figure 6B, lanes 19–21 and 25–27), indicating that the p32 interaction was not sequence-specific. Consistently, a competition assay showed that the retarded band was more strongly competed with by

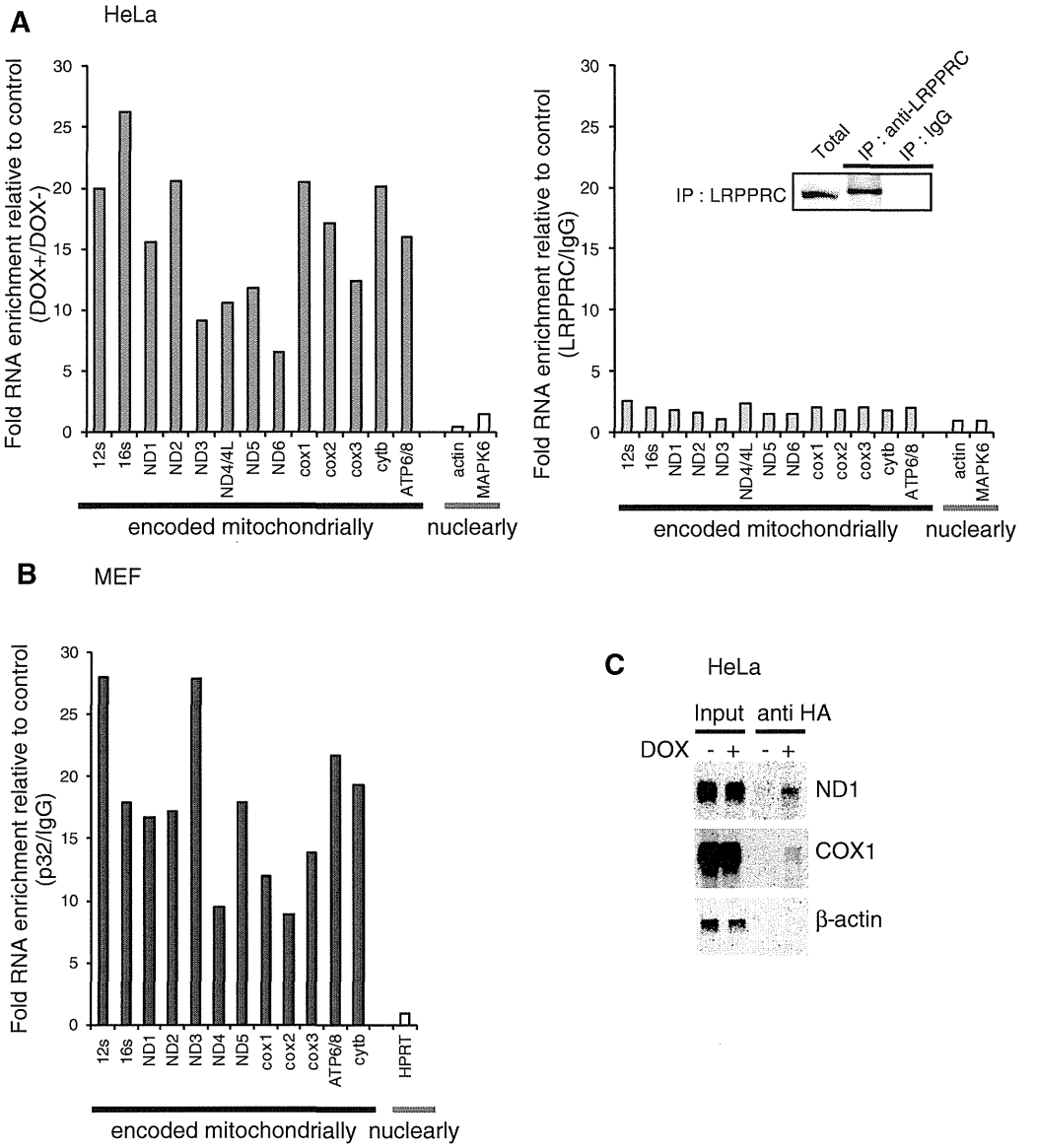

Figure 5. p32 interaction with RNA and the mitoribosome. (**A**) p32 is associated with mitochondrial RNA in HeLa cells. p32-HA expression in HeLa cells was induced with doxycycline (DOX+) or not (DOX–). HA-tagged p32 was immunoprecipitated (left panel). LRPPRC was also immunoprecipitated using the LRPPRC antibody and confirmed the IP by western blotting (right panel). The each RNA levels were measure by qPCR and the ratio of each RNA level (LRPPRC/IgG) is shown for mitochondria- and nuclear-encoded genes. (**B**) In MEF cells, p32 were immunoprecipitated using the p32 antibody. Then RNA was extracted from the immunoprecipitated samples and each RNA species was measured by qPCR as described in the materials and methods. The ratio of each RNA level (p32/ IgG) is shown for mitochondria- and nuclear-encoded genes. (**C**) Co-IP of RNA visualized on northern blot analysis in HeLa cells. RNA from whole-cell lysates (lanes 1 and 2) and immunoprecipitants with anti-HA (lanes 3 and 4) were loaded on gels. Northern blot analysis was performed using ND1, COX1 and β-actin probes.

unlabeled 40-mer RNA than by 22-mer poly(A) RNA and 14-mer RNA (Figure 6C), indicating that p32 bound to RNA with a length dependency. The retarded band was not competed with by unlabeled poly(A) DNA (Figure 6C). These results suggest that p32 binds to mitochondrial mRNAs in a manner dependent on length, but not sequence.

Lysine acetylation is a reversible post-translational protein modification. In many proteins, the acetylation plays a key role in regulating gene expression and protein function. A global analysis of lysine acetylation revealed that lysines 91 and 95 of human p32 were acetylated (46). These basic amino acid residues in the α helix H1, which is located on the edge of p32, may be able to provide electrostatic interactions with the RNA phosphate groups, raising a possibility of the involvement of the two lysines in the RNA binding. Therefore, we focused on the corresponding lysines in mouse p32, i.e. helix 1 K89 and K93 (Figure 6A, lanes 8–13). We constructed a double mutant, K89A/K93A, in which the two lysine residues were replaced by alanine. The RNA-binding activities of His-fusion wild-type and mutant p32 proteins to 22-mer poly(A) RNA, random 14-mer and random 40-mer RNAs were analyzed by a REMSA (Figure 6B, lanes 4–6, 10–12,

22–24 and 28–30). Mutant p32 showed a very weak RNA–protein complex band. The dissociation constant (K_d) of the complex for wild-type p32 was estimated to be 6 μM by a mobility-shift assay. However, the p32 lysine mutant diminished poly(A) affinity up to K_d 20 μM (Figure 6A). This mutant p32 retained the same intact trimer structure as wild-type p32 as revealed by size exclusion chromatography (Supplementary Figure S6). These results suggest that the lysine residues in α helix H1 are involved in this RNA binding.

To examine the importance of the RNA-binding activity of p32 in mitochondrial translation, we re-expressed

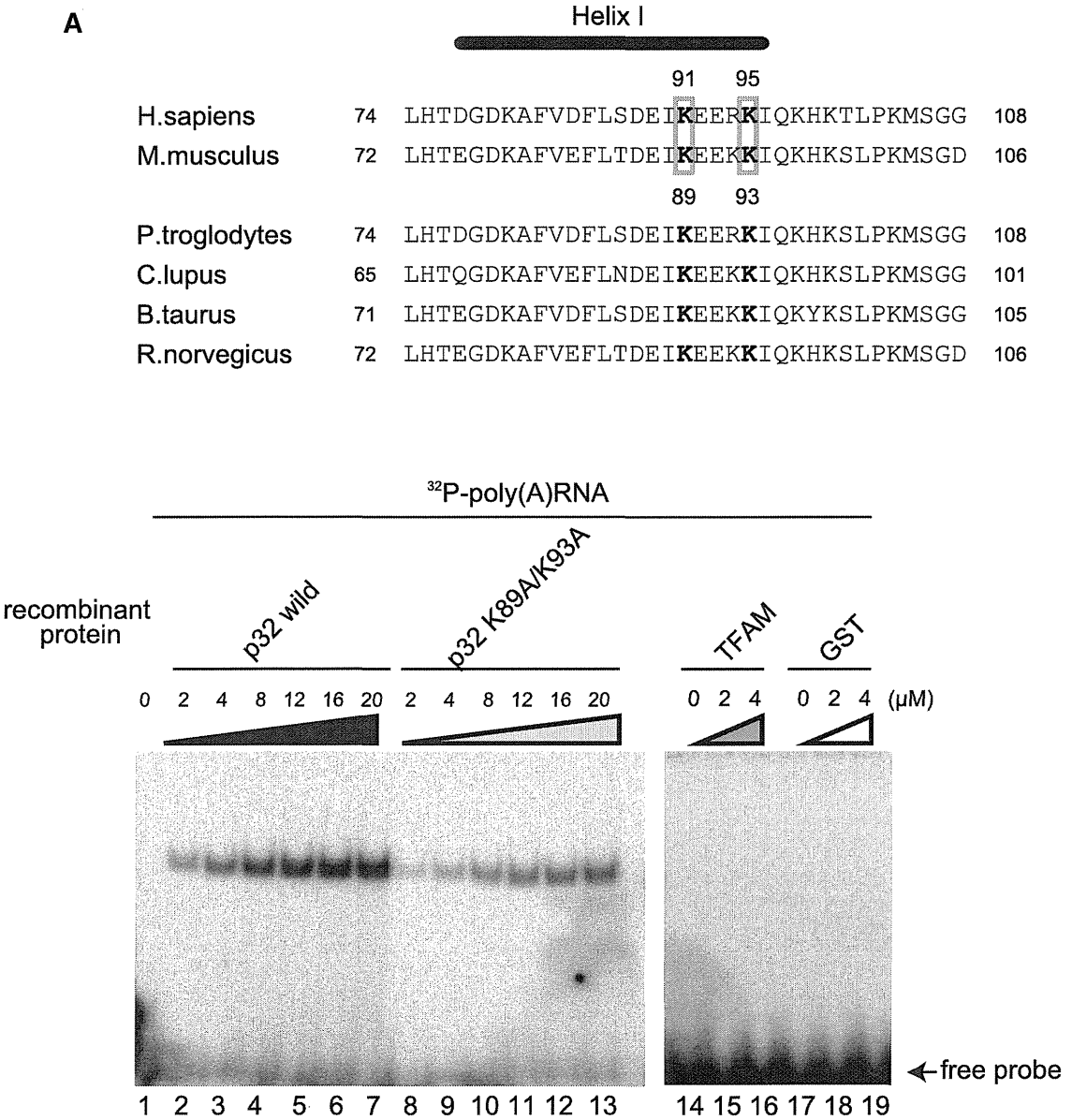

Figure 6. p32 binds to RNA oligonucleotides *in vitro*. (**A**) Upper: diagram of alignment in the α helix I of *Homo sapiens*, *Mus musculus*, *Pan troglodytes*, *Canis lupus*, *Bos taurus* and *Rattus norvegicus* p32. K89 and K93 in mouse p32 are shown as bold. Lower: purified wild-type and mutant p32, but not recombinant TFAM-His and GST proteins, bind to poly(A) oligonucleoside. Protein–poly(A) RNA complexes were separated by a REMSA using a 6% native polyacrylamide gel. An arrow indicates free probe. Recombinant wild-type and mutant proteins (2–20 μM) were incubated with 22-mer poly(A) RNA and then separated by a REMSA. Purified p32 bind to poly(A) oligonucleotide prefer to mutant p32. (**B**) The indicated amount of wild-type His-p32 and mutant His-p32 (K89A/K93A) fusion protein was incubated with ^{32}P-labeled random 14-mer RNA, 40-mer RNA, 22-mer poly(A) RNA, 22-mer poly(U) RNA or 22-mer poly(A) DNA oligonucleotides at 25°C for 30 min. p32–oligonucleotide complexes were separated by a electrophoretic REMSA using a 6% native polyacrylamide gel. The arrow indicates free probe. (**C**) Competition assays were performed by adding unlabeled RNA or DNA. The indicated fold amount of unlabeled RNA or DNA (0.2×, 1.0× or 5.0× relative to the ^{32}P-labeled probe) was incubated with recombinant p32 (4 μM) and ^{32}P-labeled poly(A) RNA; the complexes were separated by native polyacrylamide gel electrophoresis. The arrow indicates free probe. The lower panel indicates the intensity of the complex band. The 100% value represents the intensity of the complex of recombinant p32 and ^{32}P-labeled poly(A) RNA. (**D**) Expression of mitochondrial proteins. Cell lysates were prepared from equal amounts of wild-type MEFs, p32-knockout MEFs and MEFs with reintroduced wild-type *p32* cDNA, vector-only cDNA and mutant K89A/K93A *p32* cDNA. Blots were incubated with antibodies for the indicated proteins.

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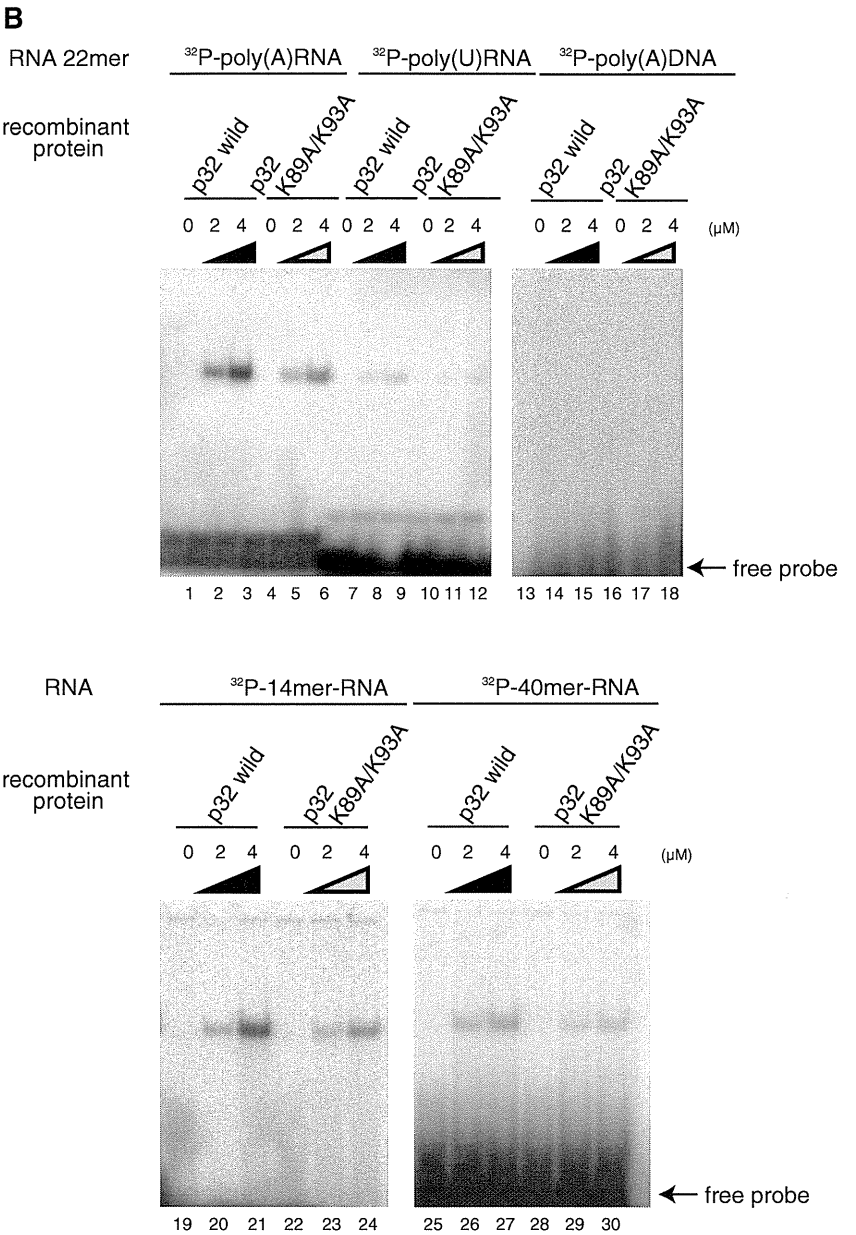

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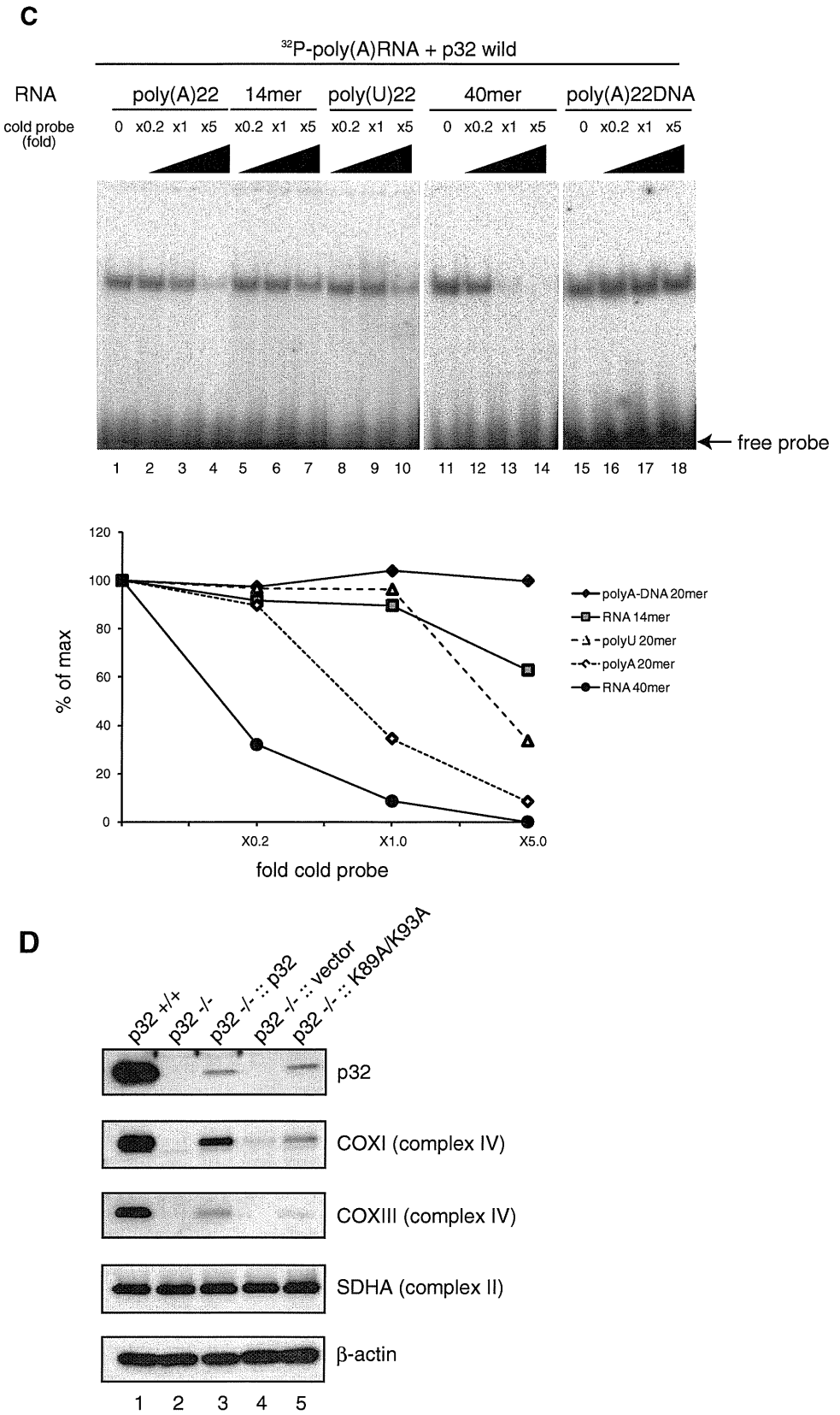
wild-type and K89A/K93A mutant p32 in p32-knockout MEFs. The two p32 proteins were expressed to a similar degree (Figure 6D, lanes 3 and 5 in the uppermost panel). p32 deficiency caused defects of mitochondrial COXI and COXIII (lanes 1 and 2). Re-expression of wild-type p32 restored COXI and COXIII (lane 3). However, re-expression of the lysine mutant p32 restored COXI and COXIII much more weakly than did the wild-type p32 (lane 5). There were no differences in the expression levels of SDHA and β-actin, indicating that the lysine residues of p32 partially affected the COXI and COXIII protein level. We also performed *in vivo* labeling using the K89A/K93A mutant and observed that translation synthesis of the p32 mutant was less than the re-expressed wild-type p32 (Figure 3D, lanes 4 and 5). The COXI restoration ability of the K89A/K93A mutant was well correlated with its RNA-binding ability (Figure 6A and D). These results suggest that the RNA-binding ability of p32 is important for mitochondrial protein synthesis in mammalian cells.

Proteins associated with human p32

To further examine the association of p32 with translating mitoribosomes, we sought proteins that interacted with p32 in mitochondria. We immunoprecipitated p32-HA-associated proteins with anti-HA antibodies after cross-linking reactions in HeLa cells (Supplementary Figure S7). The cross-linked proteins that were identified by LC–MS/MS are listed in Table 2. This analysis revealed many mitoribosomal proteins, supporting the



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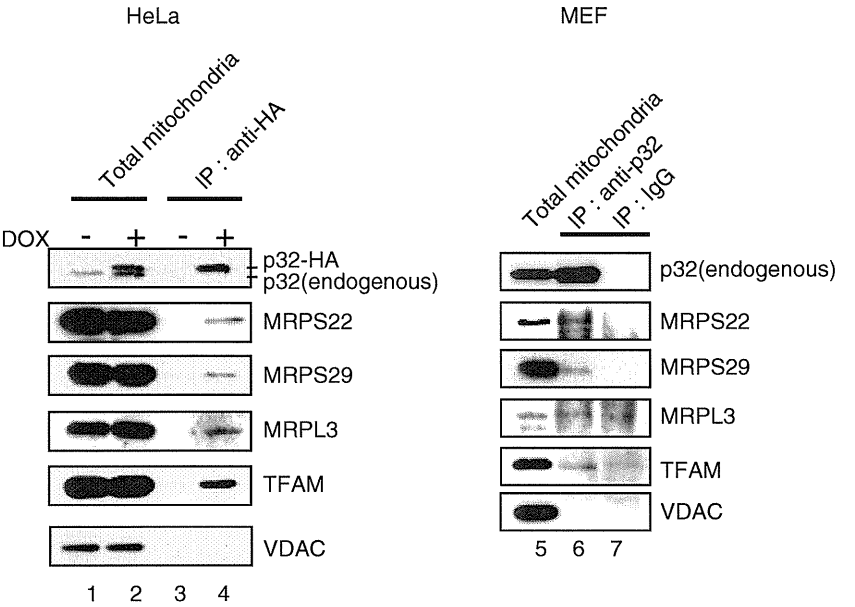

Figure 7. p32 interact with several ribosomal protein. p32-HA expression in HeLa cells was induced with doxycycline (DOX+) or not (DOX–). Lysed mitochondria were immunoprecipitated with anti-HA agarose (left panel), and a lysate of $p32^{+/+}$ MEFs was immunoprecipitated with an anti-p32 antibody (right panel) followed by western blotting with antibodies for the indicated proteins.

Table 2. List of proteins identified in complex with p32

Function	Gene				
28S ribosomal	MRPS10	MRPS2	MRPS22	MRPS23	MRPS25
	MRPS26	MRPS27	MRPS28	DAP3	MRPS30
	MRPS34	MRPS5	MRPS9	MRPS7	
39S ribosomal	MRPL12	MRPL18	MRPL19	MRPL21	MRPL22
	MRPL38	MRPL4	MRPL44	MRPL45	MRPL46
Chaperone	HSPD1	HSPA9	HSPA8	TRAP1	
Translation	ERAL1	TSFM	TUFM		
RNA binding	LRPPRC	PTCD3			
Protease	LONP1	CLPX			
Transcription	TFB2M	TFAM			
Others	PHB	PHB2			

role of p32 in the translation. In addition, many proteins that were related to nucleoids, RNA-binding and translation machinery were identified by LC-MS/MS. To confirm the association of p32 with ribosomal proteins, we carried out IP and western blotting using p32-HA-overexpressing HeLa cells and $p32^{+/+}$ MEFs. Ribosomal proteins including MRPS22, MRPS29 and MRPL3 were detected in the immunoprecipitates. However, VDAC were not immunoprecipitated with anti-p32 antibodies (Figure 7). These results suggest that p32 is associated with mitoribosomes in conjunction with all mitochondrial mRNAs for mitochondrial protein synthesis.

DISCUSSION

We have shown that p32 is essential for mouse embryonic development, based on the following evidence: (i) p32 is ubiquitously expressed in whole-mouse embryonic tissue; (ii) embryos lacking p32 are shrunken, show significantly arrested development and die at approximately E10.5; (iii) mitochondria in p32-deficient MEFs show morphological aberrations, whereas other organelles appear to be unaffected. These results suggest that mitochondrial p32 is a key molecule in mouse embryonic development.

The function of p32 in mitochondria was investigated in knockout MEFs: $p32^{-/-}$ cells. The knockout cells show severely impaired activities of electron transfer chain complexes I, III and IV but not complex II, leading strong support to requirement of p32 for expression of the mitochondrial genome. We showed that p32 deficiency impairs protein synthesis, but does not cause loss of mtDNA and transcripts. We speculated that p32 can bind to mitochondrial mRNA, but p32 is not involved in stabilizing mitochondrial mRNA. There are many mitochondria RNA-binding protein such as PTCD1~4 and LRPPRC which might be involved in stabilizing the mt-mRNA, suggesting that loss of p32 protein have no effect on the steady-state levels of mRNAs. Thus, p32 appears to be specifically required for mitochondrial protein synthesis. In sucrose gradient centrifugation and western blot experiments, p32-knockout cells exhibited normal mitoribosomal subunit assembly, but decreased 55S mitoribosomes, suggesting that p32 is not required for the subunit assembly, but is required for correct functioning of mitoribosomes with mRNA. Co-IP assays showed that p32 interacts with all mitochondrially encoded mRNAs, at least in part via poly(A) binding, and localizes close to the mitoribosome.

In this study, we obtained stable $p32^{-/-}$ MEFs that expressed a low level of mitochondrially targeted p32 (Supplementary Figure S3). Even at low-level expression in $p32^{-/-}$ MEFs, re-expression of p32 in $p32^{-/-}$ cells partially restored complex activity, mitochondrial ATP production, cell proliferation and protein translation, suggesting that a small amount of p32 rescues the

mitochondrial respiratory function via mitochondrial translational regulation. We successfully expressed p32 protein with the same plasmid up to a similar level of endogenous p32 in a human cancer cell line (data not shown), suggesting that the mechanism of p32 expression in MEFs is different from that in human cancer cells.

The Shine-Dalgarno sequence in the 5′-untranslated region of prokaryotes and the 7-methylguanylate cap structure in eukaryotic cell cytoplasm facilitate ribosome binding and direct the ribosome to the start codon. In contrast, mitochondrial mRNAs do not possess 5′-untranslated nucleotides or a cap structure (13,47). The small subunit of mitoribosomes appears to tightly bind mitochondrial mRNA in a sequence-independent manner without initiation factors or an initiation tRNA(48). However, the exact mechanisms of mRNA binding to mitoribosomes are unclear. Because of the unusual characteristics of mitochondrial mRNAs, it is thought that the mRNA 'entry gate' on the small subunit recognizes the unique unstructured 5′-sequence of mitochondrial mRNA (49,50). Here, we observed that p32 is required for the association of mitochondrial mRNAs with mitoribosomes (Figures 5 and 7). One possible mechanism is that p32 mediates the binding of mRNA to the small subunit and consequently enhance 55S formation. In prokaryotes, it is believed that initiation of leaderless mRNA occurs in intact 70S ribosomes. In *p32*$^{-/-}$ cells, the 55S mitoribosome peak was strongly reduced (Figure 4B, upper panel). Hence, another possible mechanism is that p32 contributes to guiding this unique 5′-leaderless mRNA to mitoribosomes for initiation of translation in part by enhancing the mitoribosome formation or stabilizing the mitoribosome.

We observed that the total ATP level was significantly higher in p32-knockout MEFs than in wild-type MEFs (Figure 2D), and the total ATP level is almost the same as glycolytic ATP production in p32$^{-/-}$ MEFs. The knockdown of RelA, the dominant NF-κB transactivating subunit, markedly enhances glucose consumption and lactate production in MEFs under normal culture conditions. RelA-deficient cells also exhibit decreased oxygen consumption and cell survival, although they show increased ATP levels (51), the same as p32$^{-/-}$ MEFs, suggesting that p32- and RelA-deficient cells show increased ATP levels due to the high activity of aerobic glycolysis.

Using *in vivo* labeling, we showed that re-expression of p32 in p32-deficient MEFs partially recovers the translation (Figure 3D, lane 4). However, the ratio of each protein is almost the same as the wild-type (lane 2), indicating that mtDNA-encoded protein synthesis is equally and generally suppressed and restored in p32$^{-/-}$ cells and in p32-resotred cells, respectively. Re-introduction of p32 into p32$^{-/-}$ cells well rescues complex I activity, but cannot compensate the reduced activity of complexes III and IV to a similar extent. This discrepancy may be due to the number or function of the assembly factors of each complex.

Mitochondrial RNA-binding proteins identified in previous proteomic studies include mitochondrial ribosomal proteins, tRNA synthetases, AUH, p32, LRPPRC (leucine-rich PPR motif-containing protein) and ribonuclease H1. Ponamarev *et al.* performed a proteomic analysis of bovine mitochondrial proteins with affinity to polyadenylate or polyuridylate (18). Of the 64 identified proteins, 51 possess a defined mitochondrial function, including 6 known RNA-binding proteins such as AUH. That study also showed by affinity purification that mitochondrial adenylate kinase 3, AUH and carnitine acetyltransferase bound poly(A), but not poly(U), while p32 bound poly(U) but not poly(A). However, our *in vitro* cell-free experiments demonstrated that purified recombinant p32 preferentially binds poly(A) rather than poly(U) RNA. The reason for this discrepancy is currently unknown. p32 may bind poly(U) via another protein in a crude system.

Disruption of hyaluronan synthetase 2 gene by generation Has$^{-/-}$ transgenic mice also results in abnormalities during midgestation with severe cardiac and vascular deformities, having similar phenotypic changes that takes place with p32/gC1qR/HABP1-deficient mice (52). Apoptosis induction by mitochondrial accumulation of HABP1 in fibroblast cell line with the generation of ROS and inhibition in complex I and mitochondrial dysfunction is supporting the OXPHOS regulation of p32 (53).

Acetylation of lysine is a reversible post-translational modification, which neutralizes the positive charge of this amino acid and modulates protein function in diverse ways. It plays a key role in the regulation of gene expression via the modification of core histone tails by histone acetyltransferases and deacetylases. A global analysis of lysine acetylation showed that p32 is acetylated at lysines 91 and 95 in human cell lines (46), suggesting a possible role of the lysine acetylation in p32 function and mitochondrial mRNA regulation.

Three p32 molecules form a doughnut-shaped quaternary structure with a sizable central channel and an unusual asymmetric charge distribution on the surface. This structure is highly positively charged because of the Lys residues in the α helix H1, which are located at the edge of the cleft of p32. A row of Lys residues in the α helix H1 on the surface of the molecule is conserved between human and mouse p32. The distances between these Lys residues are similar to those between the RNA phosphate groups, raising the possibility that the lysine residues continuously bind to single-stranded RNA. The alanine mutation of the two lysines in fact decreased the RNA-binding activity of p32 (Figure 6). Combined with their structural position, the lysine pair in p32 could cooperatively contribute to binding a region of single-stranded RNA. These lysine residues are conserved only among the mammals (Figure 6A) and not conserved in *Caenorhabditis elegans* or *S. cerevisiae* (23). Hence, the two lysine residues K89/K93 of the mouse p32 might play an indirect or additional role in RNA binding and resultantly in mitochondrial translation. At present, it remains yet to be identified which amino acids of p32 directly bind to RNA. However, the present lysine mutation results clearly support the importance of RNA-binding ability of p32 for the mitochondrial translation.

Mammalian mitochondria contain their own genome that is almost fully transcribed from both strands, which generates polycistronic RNA units that are processed and

matured. Mitochondrial mRNAs are modified by oligo- or polyadenylation at their 3′-termini, but the exact function of this post-transcriptional addition is unclear. The role of polyadenylation in transcription may involve mRNA stability (54). To analyze the function of polyadenylation in mitochondria, Wydro *et al.* manipulated the mitochondrial mRNA poly(A) tail by targeting a cytosolic poly(A)-modifying enzyme (PABP1) to mitochondria. The observed decline in mitochondrial translation is likely due to a dominant negative action of mtPABP1 via disruption of essential protein–protein interactions in the poly(A) extension. These results indicate that poly(A) normally interacts with endogenous components that promote translation. However, coating of the poly(A) tail by mtPABP1 did not lead to transcript decay, but caused a marked inhibition of mitochondrial translation. It was also shown that removal of the 3′-adenylyl extensions results in a variable effect on mRNA steady-state levels, increasing ND1, ND2 and ND6 mRNAs or decreasing COX1 and COX2, which suggests that the mitochondrial RNA degradosome is involved in these changes. Those data are consistent with endogenous RNA-binding factor(s) such as p32 interacting with poly(A) to optimize mitochondrial protein synthesis and increase mRNA.

p32 is a very acidic protein with a calculated isoelectric point of 4 (23). In contrast, the one side of the doughnut-shape is much less negatively charged. This polarity in charge distribution clearly suggests asymmetric functional roles for the two sides of the protein. These conserved surface features are very likely to be important for protein–protein interactions and ligand binding. We observed that various proteins, such as mitoribosomal proteins, are associated with p32. In *Escherichia coli*, the majority of ribosomal proteins in both 30S and 50S subunits are basic. The acidic side of p32 may be involved in protein–protein interactions, such as those with the mitoribosome, while the other side of p32 is involved in binding RNA via its α helix H1.

RNA chaperones are proteins that non-specifically interact with RNA and promote RNA folding by either resolving non-native conformations or impeding their formation (55). Based on our findings that p32 shows significant RNA binding and stimulates translation, we speculate that RNA chaperoning is a major activity of p32. We suggest that the putative RNA chaperone activity of p32 contributes to RNA rearrangement during the early phase of translation initiation. A chaperoning function may be important for p32 to transport RNAs to the mitoribosome.

The RNA-binding ability is well correlated with the mitochondrial protein restoration ability in $p32^{-/-}$ MEFs. Thus, the RNA-binding activity of p32 may be critically important for proper and efficient translation in the mitochondrial matrix. p32 may not only guide mRNA to the mitoribosomes, but also markedly contribute to efficient initiation and/or elongation reactions, because mitoribosomes in the heavier fractions strongly decreased in $p32^{-/-}$ MEFs. Taken together, we provide the first demonstration that p32 plays a vital role in mitochondrial homeostasis and fetal development.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1, Supplementary Figures 1–7 and Supplementary Methods.

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Research article

Different circulating brain-derived neurotrophic factor responses to acute exercise between physically active and sedentary subjects

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Running Head: Circulating BDNF and acute exercise

Abstract

Although circulating brain-derived neurotrophic factor (BDNF) level is affected by both acute and chronic physical activity, the interaction of acute and chronic physical activity was still unclear. In this study, we compared the serum and plasma BDNF responses to maximal and submaximal acute exercises between physically active and sedentary subjects. Eight active and 8 sedentary female subjects participated in the present study. Both groups performed 3 exercise tests with different intensities, i.e. 100% (maximal), 60% (moderate) and 40% (low) of their peak oxygen uptake. In each exercise test, blood samples were taken at the baseline and immediately, 30 and 60 min after the test. The serum BDNF concentration was found to significantly increase immediately after maximal and moderate exercise tests in both groups. In maximal exercise test, the pattern of change in the serum BDNF concentration was different between the groups. While the serum BDNF level for the sedentary group returned to the baseline level during the recovery phase, the BDNF levels for the active group decreased below the baseline level after the maximal exercise test. No group differences were observed in the pattern of plasma BDNF change for all exercise tests. These findings suggest that regular exercise facilitates the utilization of circulating BDNF during and/or after acute exercise with maximal intensity.

Key words: Serum BDNF, plasma BDNF, acute exercise.

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family of growth factors. In addition to its neurotrophic and synaptotrophic actions, such as the promotion of growth and survival of neurons (Aloe and Calza, 2004; Thoenen, 1995) and learning and memory (Ma et al., 1998), BDNF may play important metabotrophic roles such as the regulation of food intake (Xu et al., 2003), glucose and lipid metabolism, and energy homeostasis (Chaldakov, 2011; Nakagawa et al., 2000; Noble et al., 2011; Tsuchida et al., 2002). BDNF is present in the nervous system and peripheral tissues, and is also found in blood (Fujimura et al., 2002; Radka et al., 1996; Rosenfeld et al., 1995). Chronic treatment with subcutaneous BDNF administration significantly decreased food intake and improved the glucose uptake in skeletal muscle (Yamanaka et al., 2007) in diabetic mice, and increased glucose transporter 4 expression in normal mice (Suwa et al., 2010). In humans, the level of circulating BDNF is associated with depression (Duman, 2004), Alzheimer's disease (Tapia-Arancibia et al., 2008), obesity (Suwa et al., 2006), glucose and lipid metabolism (Levinger et al., 2008; Suwa et al., 2006), type 2 diabetes mellitus (Suwa et al., 2006) and metabolic syndrome (Chaldakov et al., 2004). Although it has been generally accepted that the neurotrophins act by paracrine or autocrine mechanisms (Davies, 1996), evidence also indicates that circulating BDNF may exert endocrine action to reveal or execute physiologic functioning.

BDNF is present in human serum and plasma, and is much more concentrated in the serum (Radka et al., 1996). Because more than 90% of blood BDNF is stored in the platelets and is released during the clotting process (Fujimura et al., 2002), serum BDNF seems to reflect both the platelet-stored BDNF and the freely-circulating BDNF in the blood, while plasma BDNF seems to reflect only the freely-circulating *BDNF* (Lommatzsch et al., 2005).

Regular exercise is well known to have many health benefits, including the prevention and improvement of obesity (Wing and Hill, 2001), type 2 diabetes mellitus (Orozco et al., 2008) and Alzheimer's disease (Heyn et al., 2004). Several animal studies have shown that $mRNA^{BDNF}$ and BDNF protein levels increase with acute and chronic voluntary wheel running in the hippocampus (Neeper et al., 1996; Gomez-Pinilla et al., 2011), and improved learning and memory (Vaynman et al., 2004). In addition, the $mRNA^{BDNF}$ and BDNF protein expression levels in skeletal muscle have been shown to be enhanced in response to muscle contraction, which is associated with enhanced lipid oxidation (Matthews et al., 2009). Collectively, these results raise the possibility that BDNF mediates, at least in part, the adaptation to exercise.

There have been several studies examining circulating BDNF responses to acute endurance exercise (Ferris et al., 2007; Gold et al., 2003; Gustafsson et al., 2009; Matthews et al., 2009; Rasmussen et al., 2009; Rojas Vega et al., 2006; Zoladz et al., 2008). In the majority of these studies, serum (Ferris et al., 2007; Gold et al., 2003; Matthews et al., 2009; Rojas Vega et al., 2006) and plasma (Gustafsson et al., 2009; Rasmussen et al., 2009) BDNF levels increased following acute exercise. On the other hand, we (Nofuji et al., 2008) and Chan et al. (2008) showed that regular physical activity affected the resting serum BDNF level. Therefore, it appears that the circulating BDNF level is affected by both acute and chronic

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physical activity. However, the interaction of acute and chronic physical activity was still unclear.

Therefore, the aim of this study was to clarify the effect of chronic physical activity on the circulating BDNF responses to acute exercise. In the present study, we simultaneously measured the serum and plasma BDNF concentrations before and after three exercise tests with different intensities for the physically active and sedentary subjects.

Methods

Subjects

Eight physically active and 8 sedentary female Japanese subjects participated in this study. "Active" was defined as performing regular sports activities more than 3 times per week for more than 3 years. The active group included distance runners (n = 3), basketball players (n = 3), and badminton players (n = 2). The sedentary subjects had not performed any regular exercise for at least 1 year. All participants were non-smokers, free from any diseases, and not taking any medications. This study was conducted in accordance with the Declaration of Helsinki, and approved by the ethics committee of the Institute of Health Science, Kyushu University, Fukuoka, Japan. Written informed consent was obtained from all participants prior to their participation.

Exercise tests

All subjects performed 3 different exercise tests in 3 separate days. At Day1, they performed the graded exercise test (maximal) to determine their volume of peak oxygen uptake (VO_2peak). After 15 min of seated rest, the subjects started pedaling at 0 W (for the sedentary group) or 30 W (for the active group). The workload was increased by 30 W in every 4 min until a 4.0 $mmol \cdot L^{-1}$ of blood lactate level was obtained. After that, the workload was increased by 15 W in every 1 min until exhaustion. "The blood for measuring the lactate concentration was obtained from an ear lobe and blood lactate level was measured using the Lactate Pro instrument (Lactate Pro LT-1710, ARKRAY, Kyoto, Japan) in every 3 min and immediately after exercise test." The heart rate (HR) was monitored using an electrocardiogram telemetry system (DS-3140, Fukuda Denshi, Tokyo, Japan). The VO_2 peak was defined as the highest VO_2 obtained during a maximal exercise test.

Two submaximal exercise tests were conducted at Day2 or 3 in random order. Trials consisted of a 30-min cycle ergometry (Monark 828E) at a constant load of 60% (moderate) or 40% (low) of the subject's VO_2peak, preceded by a 15 min of seated rest. The HR and VO_2 were recorded during each exercise test.

The subjects were instructed to refrain from heavy exercise the day before each exercise test. All exercise tests were conducted at 9:00-10:30 to diminish the effect of circadian changes in circulating BDNF levels (Piccinni et al., 2008).

Physical activity level

The daily physical activity level was evaluated with an accelerometer (Lifecorder, Suzuken Co., Nagoya, Japan). This device comprises an acceleration sensor, an amplifier, a microprocessor and memory, and was employed to ensure different physical activity levels between the two groups. All participants attached the accelerometer for 1 week just before the Day1.

Anthropometric measurements

Anthropometric measurements were conducted at Day1. The percentage of body fat was measured by bioelectrical impedance analysis device (Tanita, Tokyo, Japan).

Blood collection and biochemical analysis

In each exercise test, blood samples were taken from an antecubital vein in a sitting position at the baseline time, and immediately, 30 min and 60 min after the exercise. The blood samples were drawn into additive-free containers (serum) or heparinized containers (plasma). After kept at room temperature for 1 hour, the serum samples were centrifuged at 2000 × *g* for 10 min at 4°C. Plasma samples were immediately centrifuged. Supernatants were stored at -80°C until the analyses were performed. The serum and plasma BDNF concentrations were measured using an enzyme-linked immunoassay (ELISA) kit (Promega, Madison, WI).

Statistical analysis

The anthropometric measurements and physiological responses to maximal exercise tests between the active and sedentary groups were compared using Student's unpaired t-test. The comparisons of physical responses during moderate and low exercise tests in each group and serum BDNF level at rest between the groups were performed using the paired t-test. The changes in BDNF responses were assessed by two-way (4 time point × 2 groups) repeated measures analysis of variance (ANOVA). If an interaction was significant, one-way ANOVA was performed. A Dunnett's test was employed for all post-hoc tests. The alpha-level was set at 0.05.

Results

Characteristics of the subjects

The subject characteristics are summarized in Table 1. There were no significant differences in any anthropometric variables between the two groups. The daily physical activity level was significantly higher in the active group compared to the sedentary group ($p < 0.05$).

Table 1. Characteristics of the subjects. The data are expressed as the means (± SD).

	Sedentary	Active
Age (years)	22.8 (1.9)	21.6 (3.0)
Height (m)	1.59 (.06)	162.9 (6.8)
Weight (kg)	50.8 (6.7)	54.5 (7.5)
Body mass index ($kg \cdot m^{-2}$)	20.0 (2.0)	20.5 (1.9)
Body fat (%)	23.6 (5.9)	21.8 (2.0)
Total energy expenditure ($kJ \cdot day^{-1}$)	7451 (793)	8749 (842)**
Moving-related energy expenditure ($kJ \cdot day^{-1}$)	1115 (379)	1970 (640)**
Step count ($steps \cdot day^{-1}$)	10890 (2950)	14961 (4188)*

* $p < 0.05$, ** $p < 0.01$

Table 2. Physical parameters at the end of the low and moderate exercise tests. The data are expressed as the means (± SD).

	Low exercise		Moderate exercise	
	Sedentary	Active	Sedentary	Active
VO_2 ($ml \cdot kg^{-1} \cdot min^{-1}$)	10.8 (2.6)*	14.7 (2.4)*	20.0 (4.4)	22.9 (2.7)
%VO_2 (%)	30.8 (5.6)*	35.2 (8.0)*	58.4 (8.9)	54.8 (5.1)
Heart rate (bpm)	99 (12)*	100 (13)*	141 (16)	130 (14)
Workload (W)	39 (11)*	66 (8)*	77 (16)	107 (12)

* Significantly different from the moderate exercise ($p < 0.05$)

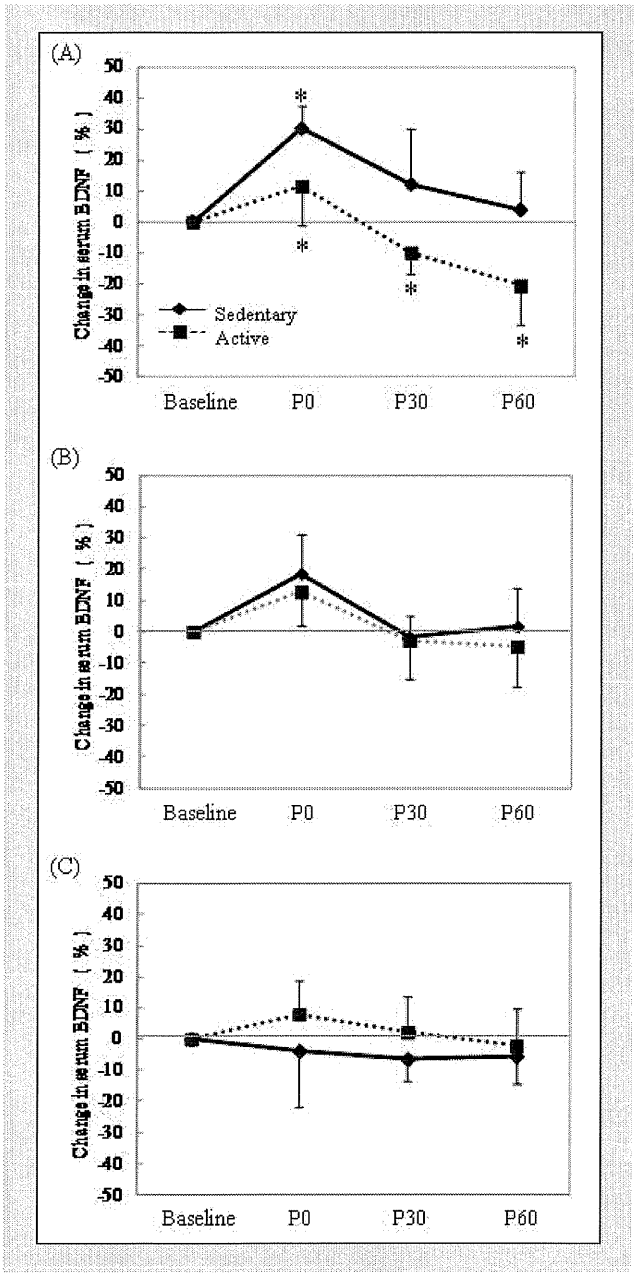

Figure1. Level of serum BDNF concentrations before maximal (A), moderate (B), or low (C) exercise tests (baseline), immediately after (P0), 30 min after (P30), and 60 min after (P60) the exercise session. The data are expressed as the means ± SD. * $p < 0.05$ vs. baseline. The changes in BDNF responses for the groups were assessed by two-way repeated ANOVA. As an interaction and main effect of time were significant, one-way ANOVA followed by a Dunnett's post-hoc test was performed.

Physical parameters in the exercise test

The VO_2peak and workload at the end of maximal exercise in the active group (42.3 ± 4.5 $ml \cdot kg^{-1} \cdot min^{-1}$, 199 ± 16 W, respectively) was significantly higher than that in the sedentary group (34.7 ± 4.0 $ml \cdot kg^{-1} \cdot min^{-1}$, 147 ± 16 W, respectively, < 0.01). There were no significant differences in the HR (Sedentary 183 ± 5 bpm, Active 179 ± 12 bpm, $p = 0.45$) and blood lactate level (Sedentary 9.6 ± 0.8 $mmol \cdot L^{-1}$, Active 8.6 ± 1.4 $mmol \cdot L^{-1}$, $p = 0.14$) between the groups at the end of the maximal exercise test. Table 2 shows the physical parameters for two submaximal exercise tests. All parameters were significantly higher at the moderate exercise test than at the low exercise test. There was no group difference in the average BDNF level at rest (Sedentary; 11.9 $ng \cdot ml^{-1}$, Active; 12.5 $ng \cdot ml^{-1}$, $p = 0.49$).

Change in the serum BDNF concentration

For the maximal exercise test, a two-way ANOVA for repeated measures on serum BDNF levels revealed significant interactions of the factors ($F(3, 42) = 7.01$, $p < 0.01$). A subsequent one-way ANOVA for repeated measures revealed a significant effect of time ($F(3, 45) = 24.8$, $p < 0.01$). The serum BDNF concentrations significantly increased immediately after the maximal exercise test in both groups (Sedentary; +30% $p < 0.01$, Active; +11% $p < 0.01$ vs. baseline, Figure 1A). While BDNF levels in the sedentary group returned to the baseline level during the recovery phase (30 min; +12% $p = 0.06$, 60 min; +4% $p = 0.80$, Figure 1A), the BDNF levels in the active group decreased below the baseline level (30 min; -15% $p < 0.01$, 60 min; -25% $p < 0.01$ vs. baseline, Figure 1A).

For the moderate exercise, neither interactions ($F(3, 42) = 0.68$, $p = 0.57$) nor the effect of groups ($F(1, 14) = 0.86$, $p = 0.37$) on the BDNF response was observed, although the effect of time was significant ($F(3, 42) = 18.7$, $p < 0.01$). The serum BDNF concentrations in both groups increased immediately after the exercise tests (+16%, $p < 0.01$ vs. baseline, Figure 1B) and returned to the baseline level during the recovery phase (30 min; -2% $p = 0.84$, 60 min; -2% $p = 0.94$ vs. baseline, Figure 1B).

The low exercise did not affect the BDNF concentration in either group (time × group; $F(3, 42) = 1.19$, $p = 0.33$, time; $F(3, 42) = 1.17$ $p = 0.33$, group; $F(1, 14) = 4.06$ $p = 0.06$, Figure 1C).

Change in the plasma BDNF concentration

For the maximal and moderate exercise test, interactions ($F(3, 42) = 1.85$, $p = 0.15$, $F(3, 42) = 1.19$, $p = 0.33$, respectively) nor the effect of groups ($F(1, 14) = 1.40$, $p = 0.26$, $F(1, 14) = 0.67$, $p = 0.43$, respectively) on the plasma BDNF response were detected. Although the effect of time were significant ($F(3, 42) = 4.24$, $p = 0.01$, $F(3, 42) = 5.40$, $p < 0.01$, respectively), a subsequent Dunnett's post-hoc test showed no significant difference in plasma BDNF between baseline and each time point

(maximal; 0min +33% p = 0.10, 30min +10% p = 0.87, 60min -9% p = 0.89 vs. baseline, Figure 2A, moderate; 0min +11% p = 0.58, 30min -11% p = 0.59, 60min -19% p = 0.17 vs. baseline, Figure 2B).

No interaction (F(3, 42) = 0.65, p = 0.59) or main effects (time; F(3, 42) = 0.77, p = 0.52, group; F(1, 14) = 0.03, p = 0.86, Figure 2C) were found in the low exercise.

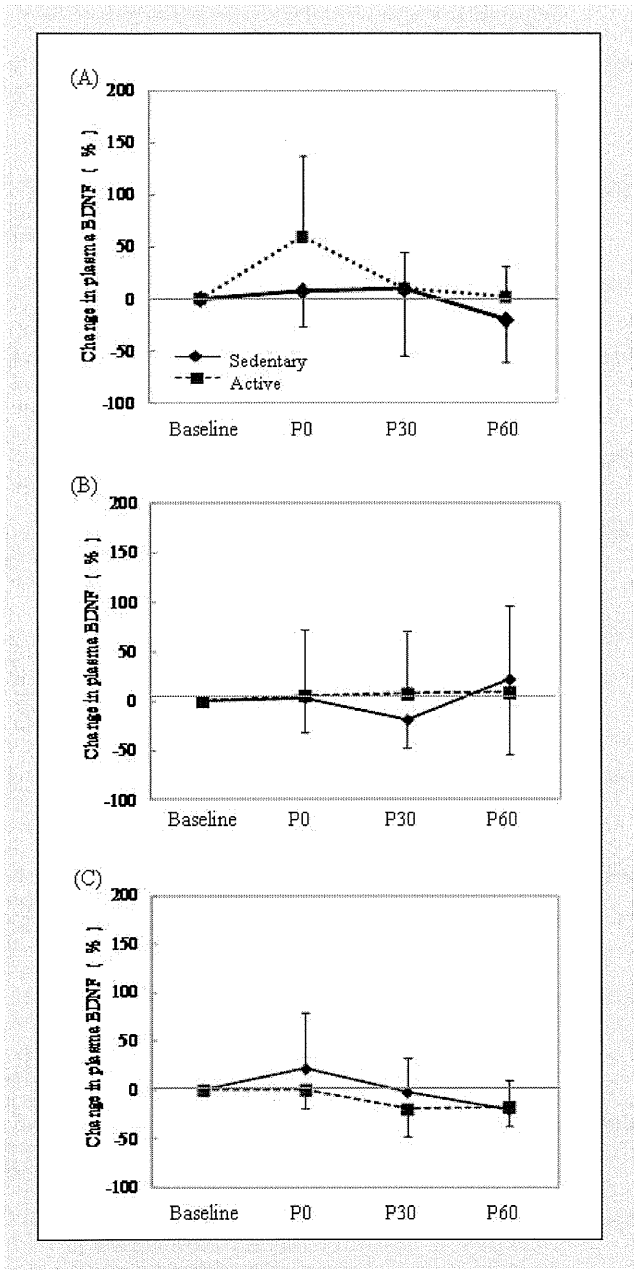

Figure 2. Level of plasma BDNF concentrations before maximal (A), moderate (B), or low (C) exercise tests (baseline), immediately after (P0), 30 min after (P30), and 60 min after (P60) the exercise session. The data are expressed as the means ± SD.

Discussion

We investigated the differences in the serum and plasma BDNF responses to acute maximal and submaximal exercises between the active and sedentary subjects. One of the novel findings of the present study was that the serum BDNF responses to maximal exercise were different between active and sedentary subjects. Especially, serum BDNF levels in the active group decreased below the baseline level during the recovery phase, while it was not the case in the sedentary group. A possible mechanism for this excessive reduction of serum BDNF in the active group is an enhanced utilization mediated by the upregulation of BDNF TrkB (tyrosine protein kinase) receptor in the peripheral tissues. Previous studies demonstrated that physical training increased the expression of TrkB in the spinal cord (Skup et al., 2000), brain (Widenfalk et al., 1999) and soleus muscle (Gómez-Pinilla et al., 2002) in rats. Although the physiological significance of the decreases in BDNF after exercise remains unknown, one of the possible roles of BDNF utilization is the repair of exercise-induced muscle damage. Ninety percent of circulating BDNF is stored in the platelets, where are also epidermal growth factor (EGF) (Oka and Orth, 1983), vascular endothelial growth factor (VEGF) (Tischer et al., 1989), and platelet-derived growth factor (PDGF) (Antoniades et al. 1979), all of which play a role in wound healing. In the current and previous studies (Ferris et al., 2007; Rojas Vega et al., 2006), serum BDNF increased with moderate- to high-intensity exercise, which has been shown to induce muscle damage (Kuipers, 1994). Therefore, it is possible that the increased BDNF during exercise contributes to the repair of skeletal muscle damaged. Although there are no direct reports demonstrating that circulating BDNF acts in the repair of exercise-induced muscle damage, BDNF treatment suppressed the release of creatine kinase and prostaglandin E2, which are common indicators of muscle cell damage in the rat muscle exposed to oxidative stress *in vivo* (Lian et al., 1998). Furthermore, the delayed regeneration of muscle fibers after injury was observed in muscle-specific BDNF knockout mice, suggesting that BDNF plays an important role in the regeneration of muscle fibers (Clow and Jasmin, 2010). Based on the potential wound-healing functions of BDNF, it is proposed that the utilization of serum BDNF during exercise may help muscle regeneration following exercise-induced damage and that the active group may have adapted to utilize circulating BDNF for the promotion of muscle repair.

Conclusion

In conclusion, the circulating BDNF responses to acute maximal exercise were different between active and sedentary groups. While serum BDNF levels in the sedentary group returned to the baseline level during the recovery phase, the BDNF levels in the active group decreased below the baseline level after high-intensity exercise. These results raise the possibility that regular exercise facilitates the utilization of circulating BDNF after acute exercise with maximal intensity. Limitations of this study were the small sample size. Additional studies with large sample size are called for. Likewise, further studies should clarify the mechanisms and physiological significance of the exercise-induced responses to circulating BDNF.

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Key points

- In maximal exercise test, the pattern of change in the serum BDNF concentration was different between the groups.
- While the serum BDNF level for the sedentary group returned to the baseline level during the recovery phase, the BDNF levels for the active group decreased below the baseline level after the maximal exercise test.
- No group differences were observed in the pattern of serum BDNF change for moderate or low exercise tests.
- No group differences were observed in the pattern of plasma BDNF change for all exercise tests.

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Normative Data for the Montreal Cognitive Assessment in a Japanese Community-Dwelling Older Population

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Key Words

Cognitive decline · Cognitive screening · Dementia · Cross-sectional study · Community-based study · Elderly · Mild cognitive impairment

Abstract

Background: Although the Montreal Cognitive Assessment (MoCA) is acknowledged as a promising neuropsychological tool, its normative data for older populations have not been established yet. The purpose of this study was to provide normative data for the MoCA in Japanese community-dwelling older people. ***Methods:*** In a Japanese town, 1,977 participants aged 65 years or older (mean age 73.6 years; male 41.3%) completed MoCA tests. After descriptive and regression analyses, normative data were developed for MoCA scores in the population. ***Results:*** The mean MoCA score observed (21.8 points) was lower than that for normal controls (27.4 points) in the original validation study of the MoCA. Additionally, 82.6% of MoCA scores fell below the standard cutoff of 26 points for detecting mild cognitive impairment (MCI). The regression analysis showed that higher age and fewer years of formal education were associated with lower MoCA scores ($p < 0.001$). Normative data for MoCA scores were presented with respect to age and education. ***Conclusion:*** This study provided normative data for the MoCA in a Japanese community-dwelling older population. This research also suggests that conventional use of the MoCA as a screening tool for MCI might be problematic in cultures different from that in which the cutoff was developed.

Introduction

Mild cognitive impairment (MCI) represents an intermediate clinical state between normal cognitive aging and Alzheimer's disease or other types of dementia [1]. Although it is not always the case, MCI has been reported to often develop into either Alzheimer's disease or other forms of dementia and, therefore, recognized as a high-risk state for dementia development [2]. In recent discussions, community-based screening of MCI is considered one of the crucial steps to enable wide-reaching interventions for preventing or slowing the onset of dementia [3].

Montreal Cognitive Assessment (MoCA) is a brief neuropsychological tool designed for screening MCI in community health care [4] and is acknowledged as a promising instrument worldwide [5–7]. Given the need for ethnic-specific versions of neuropsychological tests [8, 9], 38 versions of the MoCA are currently developed in 31 languages (www.mocatest.org). MoCA has also

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been reported to have higher sensitivity to a subtle cognitive decline than conventional tools such as the Mini-Mental State Examination [4, 10, 11]. To date, two cohort studies reported normative MoCA data in population-based samples including a multiethnic US population [12] and a Portuguese population [13]. Both studies, however, were conducted with subjects of a wide age range, and thus, the sample sizes were scarce for the older age groups.

Because older people are the primary subjects of MCI screening and subsequent interventions, their scoring characteristics on the MoCA should be examined and demonstrated with a larger sample size. This is an urgent matter, especially for a Japanese society undergoing the world's fastest aging with the highest life expectancy. Therefore, the aim of the present study was to provide normative MoCA data specific to community-dwelling older people in a Japanese town.

Materials and Methods

Participants

The present study involved analysis of data from the baseline phase of the Sasaguri Genkimon Study (SGS) conducted from May to August 2011. The SGS is an ongoing community-based prospective cohort study in a Japanese local town, Sasaguri, aiming to explore modifiable lifestyle factors causing older people to require nursing care. Subjects of the baseline study (SGS-1) were all residents of the town who were aged 65 years or older and not certified as individuals requiring nursing care by the town in January 2011 (n = 4,979). Sixty-six subjects were excluded due to being dead or moving out by the onset of the study. A set of study information sheets and a questionnaire were mailed to all remaining subjects (n = 4,913), and 2,629 individuals, hereafter referred to as the participants of the SGS-1, responded to the mail by (1) visiting a community center to submit the questionnaire and undergo multiple physical and cognitive tests in one of 31 group-testing sessions of the SGS-1, (2) contacting study coordinators to set up an appointment for an individual home-testing session or (3) visiting the city office to submit the questionnaire (recruitment rate: 53.5%). Of these, 2,129 individuals took part in the MoCA tests. After the testing, we excluded 32 individuals who were unable to complete the MoCA properly, 12 individuals with missing information about their years of formal education, and 108 individuals with self-reported medical histories of stroke, depression, Parkinson's disease and dementia. Accordingly, data from 1,977 participants (75.2% of the total participants of the SGS-1) were involved in the present study.

Standard Protocol Approvals, Registrations and Patient Consents

All the participants provided written informed consent to participate in the present study. The study protocol and the informed consent form were approved by the Institutional Review Board of the Institute of Health Science, Kyushu University.

Measurements

We used the Japanese version of the MoCA for all measurements. The details of the Japanese version are described elsewhere [5]. Briefly, it was developed and validated by investigators, including the inventor of the original MoCA (Dr. Nasreddine). As in the original one [4], the Japanese version of the MoCA was designed as a 30-point screening instrument administered in about 10 min and consists of the following 12 cognitive tasks: a five-item delayed recall task (5 points), a clock-drawing task (3 points), a cube-copying task (1 point), a trail-making task (1 point), a phonemic fluency task (1 point), a two-item verbal abstraction task (2 points), a target-tapping task (1 point), a serial subtraction task (3 points), a two-item digits-reading task (2 points), a three-item naming task (3 points), a two-item sentence-repeating task (2 points) and a six-item temporal and locational orientation task (6 points). In the standard procedure of the original as well as the Japanese versions, 1 point is added to the total score of the cognitive tasks if an individual has 12 years or fewer of formal education, and a final total score falling below 26 points is judged to have probable MCI.

Procedures

All MoCA tests were administered to the participants by trained personnel as part of the group-testing and home-testing sessions of the SGS-1. After the testing, MoCA scores were independently evaluated by two authors (K.N. and T.H.) and double-checked between the two before being finally determined. The interevaluator reliability, shown as a percentage of agreement in the MoCA scores, was 93.3% in the initial evaluation. To demonstrate normative data in participants with a wide range of years of formal education, the preferred 1-point correction for education was not adopted.

Statistical Analyses

All statistical analyses were conducted using SAS version 9.2 (SAS Institute Inc., Cary, N.C., USA). The Wilcoxon rank-sum test and the χ^2 test were conducted to compare age and sex, respectively, between the participants of the present study and the rest of the subjects (n = 2,936). The Wilcoxon rank-sum test was also performed to assess the difference in years of formal education between the participants of the present study and the rest of the participants of the SGS-1 answering educational history in the questionnaire (n = 608). Descriptive statistics were calculated for MoCA scores and for scores of respective cognitive tasks. A multiple regression analysis was performed with the MoCA score as a dependent variable and age, sex and years of formal education as independent variables. Additionally, to visualize changes in MoCA scores, simple regression analyses were conducted between the MoCA score and age in three education levels ($\leq$9, 10–12, and $\geq$13 years of formal education). Subsequently, normative data for MoCA scores in the community-dwelling older population were developed with respect to age and education. Overlapping age categories of 65–75, 70–80, 75–85, and $\geq$80 years, accompanied by the aforementioned three education levels, were adopted in the normative data based on the rationale previously described for practical use of the normative data in community health care [12, 14]. A significance level was set at two-sided $\alpha = 0.05$.