Regulation of p68 RNA Helicase by Calmodulin and Protein Kinase C*

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Human p68 RNA helicase is a nuclear RNA-dependent ATPase that belongs to a family of putative helicases known as the DEAD box proteins. These proteins have been implicated in aspects of RNA function including translation initiation, splicing, and ribosome assembly in a variety of organisms ranging from Escherichia coli to humans. While members of this family are believed to function in the manipulation of RNA secondary structure, little is known about the regulation of these enzymes. By immunological methods and sequence comparison, we have found that p68 possesses a region of sequence similarity to the conserved protein kinase C phosphorylation site and calmodulin binding domain (also known as the IQ domain) of the neural-specific proteins neuromodulin (GAP-43) and neurogranin (RC3). We report that p68 is phosphorylated by protein kinase C in vitro and binds calmodulin in a Ca2+-dependent manner. Both phosphorylation and calmodulin binding inhibited p68 ATPase activity, suggesting that the RNA unwinding activity of p68 may be regulated by dual Ca2+ signal transduction pathways through its IQ domain.

Neuromodulin (GAP-43) and neurogranin (RC3) are neural-specific calmodulin (CaM) binding proteins shown to be phosphorylated by protein kinase C (PKC) in vitro and in vivo. Although the overall sequence homology between these proteins is low, they share a nearly identical region of 19 amino acids, corresponding to the overlapping site of CaM binding and PKC phosphorylation, also referred to as the IQ domain (1–6). CaM binding at the IQ domain of neuromodulin has been shown to decrease the rate of phosphorylation by PKC, and phosphorylation conversely prevents binding to CaM, leading to the hypothesis that reversible phosphorylation of neuromodulin at the IQ domain may regulate free CaM levels in neurons (reviewed in Ref. 7). It has recently been reported that several other proteins contain sequences related to the IQ domain (reviewed in Refs. 6 and 8). While these proteins exhibit varying degrees of similarity within the conserved domain, this site has been implicated as a region for interactions with Ca2+-binding proteins. For example, calcium vector protein target interacts with a CaM-like protein, the calcium vector protein, at the IQ domain (9). In addition, p190 (myosin-V) binds CaM at tandem repeats of the IQ motif (10). It has been proposed that this sequence represents a common descriptor for Ca2+-regulation via CaM or other Ca2+-binding proteins (8).

In this study, we identify the p68 RNA helicase as a member of the IQ domain-containing family. p68 belongs to a rapidly growing family of proteins (DEAD box family) which share a core region of highly conserved sequence motifs, including sites for ATP binding and hydrolysis (reviewed in Ref. 11). These proteins are involved in diverse cellular processes including RNA splicing, translation initiation, ribosome assembly, and cell growth and division. They are hypothesized to regulate RNA structure and function by unwinding double-stranded RNA or by promoting other ATP-dependent conformational changes. Members of the DEAD box family include the prototypic eukaryotic translation initiation factor eIF-4A and the yeast splicing factor Prp8 (11). Human p68 was first identified by its immunological cross-reactivity with the viral helicase, SV40 large T antigen (12). While the precise function of p68 is unknown, it has been shown to exhibit RNA-dependent ATPase activity and RNA unwinding activity in vitro (13–15). The presence of an IQ domain within the primary sequence suggests that the RNA helicase activity of p68 may be regulated by Ca2+ via CaM and/or by PKC. Here we report that p68 is a substrate for PKC in the absence of RNA and that phosphorylation inhibits RNA stimulation of p68 ATPase activity. In addition, CaM binds to p68 in a Ca2+-dependent manner and also blocks p68 ATPase activity. We propose that p68 helicase activity may be regulated by PKC and Ca2+ through its IQ domain.

EXPERIMENTAL PROCEDURES

Cell Culture—PC12 cells were maintained as described (16). Chinese hamster ovary (CHO) cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Life Technologies, Inc.) in a humidified 37 °C atmosphere with 5% CO2.

Protein Isolation and Antibody Production—The cDNA encoding neurogranin was subcloned into the BamHI/EcoRI sites of the bacterial expression plasmid pGEX-2T (Pharmacia Biotech Inc.). Glutathione S-transferase/neurogranin fusion protein was purified according to the method of Smith and Johnson (17). Rabbit polyclonal antibody was raised against the fusion protein, and IgG was isolated on protein A-Sepharose 4B. Western Blot Using Anti-neurogranin Antibody—Adult rat brains were homogenized in 40 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol, and the homogenate was fractionated by centrifugation at 14,000 × g for 30 min at 4 °C. CHO cells were scraped from 10-cm Petri dishes and pelleted at 2000 × g for 10 min. The pelleted cells were homogenized in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM PMSF on ice. Unbroken cells and nuclei were removed by centrifugation at 4000 × g for 2 min, and the cell extracts were fractionated by centrifugation at 100,000 × g for 30 min at 4 °C. The supernatants were collected, and protein concentration was assayed by the BCA method (Pierce). Proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose. The membrane was treated with 3% bovine serum albumin in phosphate-buffered saline containing 0.2% Tween 20 to block nonspecific binding sites and incubated overnight at 4 °C with polyclonal antibody IgG (3 μg/ml). Bound antibody was detected using alkaline phosphatase-conjugated secondary antibody (Cappel) according to the manufacturer's instructions (Bio-Rad). IgG specific to the CaM binding domain of neurogranin and neuromodulin was removed by
incubating the total IgG fraction with a molar excess of a biotinylated 16-amino acid peptide corresponding to the CaM binding domain of neuromodulin (2) and immobilized avidin (Pierce) for 60 min at room temperature. The resin was pelleted, and the depleted IgG pool was used for Western analysis as above.

**Peptide Sequencing of p68—**PC12 cells (5 x 10⁶ cells) were lysed with phosphate-buffered saline, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS for 10 min on ice. The extract was clarified by centrifugation at 14,000 x g for 10 min and precored with protein A-agarose (Oncogene Science) for 30 min at 4 °C. p68 was immunoprecipitated by rotation at 4 °C with anti-neurogranin IgG and excess protein A-agarose overnight. The immunoprecipitates were washed, separated by 10% SDS-PAGE, and electrophoresed to nitrocellulose. After detection with Ponceau S, p68 was excised and digested in situ with trypsin (20). Cleavage fragments were separated on an Applied Biosystems model 122 microbore high pressure liquid chromatograph and subjected to amino acid sequencing using an Applied Biosystems model 477A protein microsequencer.

**Biotinylated Calmodulin Binding—**PC12 cells (3 x 10⁶ cells) were lysed with 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 1% Nonidet P-40, 1 mM dithiothreitol, and 1 mM PMSF for 30 min on ice and clarified by centrifugation at 14,000 x g for 10 min. p68 was immunoprecipitated with the monoclonal antibody PAb204 as described (15), resolved by 10% SDS-PAGE, and transferred to PVDF membrane. Proteins were detected by Coomassie Blue staining as described previously (21). CaM binding was assayed according to Billingsley et al. (22), using 4 mM biotinylated CaM (Life Technologies, Inc.) and alkaline phosphatase-conjugated avidin (Pierce). Reacted samples were stopped by the addition of Laemmli buffer, resolved by SDS-PAGE, and subjected to autoradiography.

**ATPase Assay—**p68 was immunoprecipitated with PAb204 and treated with or without RNase A (0.1 mg/ml) in lysis buffer for 1 h at 4 °C, and beads were washed into PKC phosphorylation buffer. Phosphorylation assays were carried out at 30 °C with PKC in the presence of absence of effectors for times indicated according to Apel et al. (5). Reactions were stopped by the addition of Laemmli buffer, resolved by SDS-PAGE, and subjected to autoradiography.

**RESULTS AND DISCUSSION**

**An Antibody against Neurogranin Cross-reacts with p68 RNA Helicase—**In an attempt to identify proteins containing the IQ domain, polyclonal antibodies were raised against neurogranin as a fusion protein with glutathione S-transferase. Western blot analysis of rat brain extracts using anti-neurogranin IgG showed cross-reactivity with neuromodulin and a higher apparent molecular mass species of approximately 68 kDa, also detectable in CHO cell extracts (Fig. 1). The cross-reactivity with neuromodulin was presumably due to the conserved 19-amino acid sequence which contains the IQ domain, the site of CaM binding and phosphorylation by PKC. The only sequence homology between neurogranin and neuromodulin lies within this common CaM binding domain. Specific removal of antibodies against the IQ domain of neurogranin and neuromodulin eliminates the cross-reaction with neuromodulin and the 68-kDa protein (Fig. 1), indicating that a similar epitope may reside within the higher molecular mass protein. This was further supported by the finding that immunoprecipitation of the 68-kDa protein from PC12 cells with anti-neurogranin IgG was blocked in the presence of a molar excess of peptide corresponding to the IQ domain (data not shown).

In order to identify the cross-reacting protein, the 68-kDa protein was immunoprecipitated from PC12 cells and subjected to in situ trypsic digestion. Subsequent sequence analysis of six of the resultant peptides revealed significant homology with the deduced amino acid sequence of the human p68 RNA helicase (Table I) (13, 24). Western blot analysis and immunoprecipitation using the p68-specific antibody PAb204 (12) confirmed the identity of this protein (data not shown). Further examination of the primary sequence indicated that p68 contains a six-amino acid region, GFGAP, corresponding to IQASFR found within the consensus domains of neurogranin and neuromodulin. Phosphorylation of the neurospecific proteins occurs on the serine within this region (3, 5). In p68 this site is COOH-terminal to the epitope for PAb204 shared with
the SV40 large T antigen (13) and presumably constitutes the site of recognition for the anti-neurogranin antibody.

p68 RNA Helicase Binds CaM—The presence of the IQ domain in p68 suggested that the enzyme may be regulated by Ca2+-dependent processes via CaM and/or PKC. Binding of CaM to p68 was examined with a CaM overlay procedure in Ca2+-dependent binding to its target, which exhibits Ca2+-dependent binding to its target (9).

p68 Is Phosphorylated by PKC in the Absence of RNA—The conserved serine within the IQ domain of p68 corresponding to the phosphorylation site in neuromodulin and neurogranin led us to examine the phosphorylation of p68 by PKC in vitro (Fig. 3A). When immunoprecipitated from PC12 cells without further treatment, p68 was a poor substrate for PKC. However, p68 co-immunoprecipitates with RNA when cells are lysed at physiological salt concentrations (15). Removal of bound RNA with RNase A resulted in a marked increase in Ca2+- and phospholipid-dependent phosphorylation of p68 by PKC (Fig. 3A). Inclusion of CaM in the assay blocked phosphorylation of p68, with no effect on PKC autophosphorylation. CaM also inhibits the PKC phosphorylation of both neurogranin and neuromodulin (2, 5). In addition, a 20-amino acid peptide corresponding to residues 549–568 of p68 was phosphorylated in a Ca2+- and phospholipid-dependent manner by PKC (data not shown). These data indicate that a site of PKC phosphorylation of p68 lies within the conserved IQ domain; it is not known whether additional sites may be subject to PKC phosphorylation. Because p68 used in these assays was immunoprecipitated, stoichiometry of phosphorylation could not be determined. Phosphorylation of p68 was saturable, reaching maximal level by approximately 1 h (Fig. 3B).

Phosphorylation and CaM Binding Inhibit ATPase Activity of p68—Since p68 helicase is a substrate for PKC in the absence of RNA in vitro, it was of interest to determine whether phosphorylation affected the RNA-dependent ATPase activity of the enzyme. Immunoprecipitated PC12 p68 exhibited ATPase activity due to the presence of bound RNA which co-immunoprecipitates with the protein (15) (Fig. 4A). ATPase activity was almost completely abolished by RNase treatment but was re-

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**Fig. 3.** *In vitro phosphorylation of p68 RNA helicase by PKC. A*, phosphorylation of immunoprecipitated p68. p68 was immunoprecipitated from PC12 cells with PAh204 and treated with or without RNase A (0.1 mg/ml) for 1 h at 4 °C. Phosphorylation assays were carried out with PKC for 10 min at 30 °C; calcium (400 μM) free together with 200 μg/ml phosphatidyl-i-serine and 20 μg/ml diolein (designated PL) were added as indicated. Where shown, CaM (3 μM) was preincubated with p68 for 15 min prior to initiation of the phosphorylation assay. Reactions were stopped by the addition of Laemmli buffer, and phosphoproteins were separated by SDSPAGE and detected by autoradiography. B, time course of phosphorylation. Immunoprecipitated RNase-treated p68 was incubated with PKC for the times indicated. Autoradiographs were analyzed by densitometry and presented as percent maximal phosphorylation relative to the phosphorylation level at 120 min. Points shown are the mean ± S.E. of three experiments.

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**Fig. 4.** Effect of PKC phosphorylation and CaM binding on the ATPase activity of p68. A, inhibition by PKC phosphorylation. Immunoprecipitated p68 was treated with or without RNase A as described in Fig. 3. The samples were washed, and p68 was incubated with PKC in the presence or absence of CaCl2 and phospholipids for 60 min at 30 °C. After additional washing of the beads, ATPase activity was determined in the presence or absence of exogenous total RNA as described under "Experimental Procedures." B, inhibition by CaM binding. RNase-treated p68 was preincubated with ATPase buffer containing 1 μM CaCl2 in the presence or absence of CaCl2 (3 μM) for 2 h and assayed for ATPase activity as above. All reactions were performed in triplicate; each data point is the mean ± S.D. Results shown are representative of at least four experiments corrected for spontaneous ATP hydrolysis.
stored with subsequent addition of exogenous RNA (80 pg/ml). However, when p68 was phosphorylated following RNase treatment, ATPase activity was not fully recovered by the addition of exogenous RNA (~40% of maximal), illustrating that PKC phosphorylation blocked RNA stimulation of ATPase activity. This may result from reduced affinity of p68 for RNA; at higher concentrations of exogenous RNA (i.e. 0.2 mg/ml), PKC phosphorylation had little or no effect on RNA-stimulated ATPase activity (data not shown).

Interestingly, Ca²⁺/CaM also significantly inhibited the ATPase activity of the enzyme (Fig. 4B), whereas Ca²⁺ or CaM alone had no effect (data not shown). Ca²⁺/CaM markedly reduced ATPase activity of enzyme co-immunoprecipitated with bound RNA and prevented phosphorylation of p68 by addition of exogenous RNA (80 pg/ml). The inhibition of RNA-stimulated ATPase activity by CaM was concentration-dependent (Fig. 5) and suggests that the affinity of p68 for CaM lies in the low micromolar range. Although relatively high concentrations of CaM were required for inhibition of p68 ATPase activity, nuclear CaM levels have been estimated to be as high as 500 nM (26–28). Since ATPase activity is required for p68 unwinding activity, the RNA helicase activity of p68 may likewise be inhibited by PKC and/or by Ca²⁺/CaM.

The molecular mechanism for PKC or CaM inhibition of p68 RNA-stimulated ATPase activity has not yet been established, although there are several possibilities. For example, conformational changes caused by phosphorylation or by CaM binding may indirectly disrupt RNA binding, ATP binding, and/or hydrolysis. Alternatively, the phosphorylated residue(s) may reside within or near the RNA-binding site, resulting in electrostatic repulsion of nucleic acid. While the RNA-binding site of p68 is currently undefined, it has been proposed that the basic region at the carboxyl terminus of p68 is a specific RNA-binding motif (29). Moreover, the HRIGXXR region conserved in DEAD box proteins has been reported to be required for RNA binding and ATP hydrolysis of family member eIF-4A (29).

These data represent the first report of PKC/Ca²⁺ regulation of a member of the DEAD box family and strongly suggest that regulation is mediated through the IQ domain. Sequence examination of the DEAD box proteins reveals that other family members including Dhp73D (30), vasa (31), and Prp5 (32) also contain sequences similar to the IQ domain, suggesting that these proteins may also be modulated by PKC or CaM binding. Whereby the specific functions of p68 are unknown, the enzyme has been shown to undergo dramatic changes in nuclear localization during the cell cycle. It is localized in the nucleoplasm during interphase and translocates to the nucleoli during telophase, suggesting a role for p68 in nuclear assembly (33). This observation together with our results suggests that the RNA helicase activity of p68 may be regulated by PKC and/or CaM during the cell cycle. Interestingly, CaM has been implicated as the mediator of Ca²⁺-dependent regulation of cell cycle progression, especially at the G1/S, G2/M, and metaphase/anaphase transitions (for review see Ref. 34). Furthermore, Ca²⁺/CaM has been shown to inhibit nuclear events such as transcription through direct protein-protein interaction with transcription factors of the basic helix-loop-helix group (28). In conclusion, we have defined a potential mechanism for regulation of RNA secondary structure by Ca²⁺ signal transduction pathways.

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