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Mutations in a CCHC Zinc-Binding Motif of the Reovirus σ3 Protein Decrease Its Intracellular Stability

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It has been demonstrated that the σ3 protein of reovirus harbors a zinc-binding domain in its amino-terminal portion. A putative zinc finger in the CCHH form is located in this domain and was considered to be a good candidate for the zinc-binding motif. We performed site-directed mutagenesis to substitute amino acids in this region and demonstrated that many of these mutants, although expressed in COS cells, were unstable compared with the wild-type protein. Further analysis revealed that zinc-binding capability, as measured by retention on a zinc chelate affinity adsorbent, correlates with stability. These studies also allowed us to identify a CCHC box as the most probable zinc-binding motif.

The reovirus σ3 protein, encoded by the S4 gene, is a 41-kDa polypeptide which plays multiple roles during the viral multiplication cycle. It is involved in the correct processing of the viral μ1 protein to μ1C (31), forming σ3-μ1C complexes which constitute the major component of the outer capsid of mature reovirus (16, 36). In addition to this structural role, σ3 has been reported to be involved in the inhibition of host cell RNA and protein synthesis (29), in the establishment of persistent reovirus infection (1), in the stimulation of late viral mRNA translation (19), and in the inhibition of interferon action (14).

Previous studies have shown that σ3 consists of two independent functional domains (27). The carboxy-terminal domain has affinity for double-stranded RNA, apparently through two basic alpha-helical motifs (25, 27). This property of σ3 is involved in translational regulation through inhibition of the double-stranded RNA-activated protein kinase (9, 20, 22, 28). The other domain, the amino-terminal portion of σ3, is able to bind a zinc atom and also exhibits a region of similarity with picornavirus proteases (27). The function of the zinc-binding sequence has never been investigated and is the subject of the present study.

To study the function of the zinc-binding motif, we first introduced a premature termination codon at amino acid 218 (mutant ΔC) to produce the amino-terminal domain encompassing the zinc-binding region. We also created a separate mutant encoding only the carboxy-terminal domain (mutant ΔN) by deletion of the first 172 amino acids. To ensure efficient initiation of translation, nucleotides around the internal in-frame initiation codon at position 173 were mutated according to Kozak's consensus sequence (17).

Site-directed mutagenesis was performed by standard procedures. Briefly, the S4 gene was introduced into an M13 vector and the recovered single-stranded DNA was used as a template for oligonucleotide-directed mutagenesis (30, 35) as specified by the manufacturer of the kit (Amersham). Mutated fragments were then recovered from M13 double-stranded replicative forms and substituted for the corresponding fragment in a plasmid expression vector suitable for transient expression of σ3 in COS cells. In this vector, the S4 gene is placed under the control of the simian virus 40 early promoter. The plasmid also harbors the simian virus 40 replication origin to ensure replication in COS cells (10, 24).

Each mutant was transfected into COS cells by a standard calcium phosphate coprecipitation procedure (11). Transfected COS cells were labeled for 1 h with [^{35}S]methionine at 48 h posttransfection. Labeled proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) followed by autoradiography of the dried gel by standard procedures. These experiments showed that the mutant ΔN, encoding only the carboxy-terminal domain of σ3, was not expressed (data not shown). The mutant ΔC, encoding only the amino-terminal domain of σ3, was expressed but only at a low level. Furthermore, coexpression of full-length σ3 with either deletion mutant allowed better expression of only the ΔC mutant, which approached levels seen with wild-type σ3, whereas coexpression had no effect on the ΔN mutant (data not shown). This is consistent with the ability of full-length σ3 to stimulate translation of plasmid-encoded mRNAs under transfection conditions. We and others have previously demonstrated that this is due to a regulatory effect on translation that can be supplied in *trans* (2, 20, 21b, 22, 28). In contrast, the results obtained with the mutant lacking the amino-terminal domain (mutant ΔN) show that this region is required in *cis*.

To further understand the role of the amino-terminal portion of the σ3 protein, encompassing the zinc-binding region, we performed individual amino acid substitutions in this region. These mutations fell into two broad classes. The first class constitutes amino acid substitutions of different residues of the putative CCHH zinc finger motif proposed by other investigators (27), to form mutants C51A, H70–71L, H67A, and C51–54S. The second class of mutations consists of amino acid substitutions around this zinc finger sequence, to form mutants G46–47A and C73A. The positions of all these mutations on the linear amino acid sequence of σ3 are summarized in Fig. 1. Analysis by metabolic radiolabeling showed that expression of polypeptide σ3 in transfected COS cells varied among the mutants. Two different phenotypes were observed. First, mutant H67A was expressed at wild-type levels (Fig. 2); the same result was also obtained with the mutant G46–47A (data not shown). A second phenotype was found with mutants C51A, C51–54S, H70–71L, and C73A, all of

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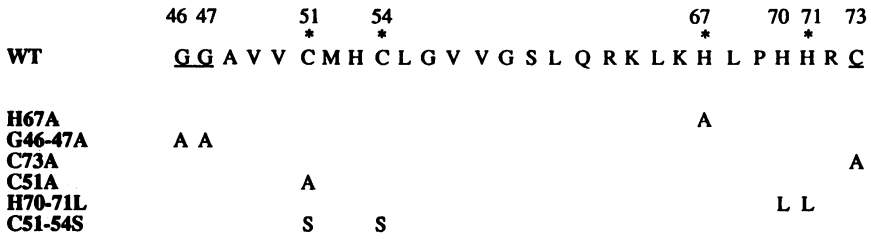

FIG. 1. Sequence of the putative zinc-binding site in the σ3 protein. The amino acid sequence is represented by the single-letter amino acid code. Residues of the putative zinc finger are marked with asterisks. Other substituted amino acids surrounding the proposed zinc finger are underlined. Names of mutants used in this study are indicated below the wild-type (WT) sequence. In mutation designations, the first letter and number represent the position of the initial amino acid and the second letter designates the substituted amino acid. The numbers refer to the amino acid positions.

which exhibited a reduced level of expression compared with the wild type (Fig. 2).

To determine if synthesis, or, rather, accumulation, of σ3 protein is affected by these mutations, we proceeded to examine the steady-state levels of the corresponding proteins. The same protein samples were thus further analyzed by electrotransfer onto nitrocellulose filter (32) followed by immunoblotting with a polyclonal rabbit anti-reovirus antiserum (Lee Biomolecular). Filter-bound antigen-antibody complexes were then revealed by using an alkaline phosphatase-conjugated anti-rabbit antibody followed by detection with Nitro Blue Tetrazolium/5-bromo-4-chloro-3-indolylphosphate toluidinium salt (NBT/BCIP) color development reagent as recommended by the manufacturer (Gibco/BRL). This experiment showed that mutant C73A exhibited a reduced expression level. However, the quantitative effect appeared more drastic when analyzed by immunoblotting compared with the previous labeling experiment (Fig. 2; compare Western blot with labeling). The same result was obtained with mutants C51A, C51–54S, and H70–71L (data not shown). These results suggest that the accumulation of σ3 proteins (detected by immunoblotting), rather than their synthesis (detected by labeling), was affected by the mutations. It has to be mentioned that we now have a collection of more than 12 mutants with point mutations, substituting alanine for different amino acids scattered throughout the σ3 molecule (21a). We have never observed a drastic decrease in the accumulation of the mutated proteins in transfected cells, as is observed with the four mutants reported here (C51A, C51–54S, H70–71L, and C73A).

To determine whether the reduced accumulation of σ3 can be directly attributed to decreased stability, we next performed pulse-chase experiments. Transfected COS cells were pulse-labeled for 30 min at 42 h following transfection, the labeling medium was then removed, and the cells were recovered immediately (time zero) or reincubated for 0.5, 5, or 24 h in fresh Dulbecco modified Eagle medium containing unlabeled methionine. Cells were then lysed in RIPA buffer (10 mM Tris-HCl [pH 7.4], 1 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, 0.2% phenylmethylsulfonyl fluoride), and proteins were subjected to immunoprecipitation with the polyclonal rabbit anti-reovirus antiserum. Immunoprecipitates were analyzed by SDS-PAGE. Results showed that mutant H67A turnover is negligible for up to 24 h, as is observed for wild-type σ3 (Fig. 3). The same result was obtained with mutant G46–47A (data not shown). In contrast, mutant C73A exhibited rapid turnover (Fig. 3). By using densitometric analysis (Molecular Dynamics Personal Laser Densitometer), the half-life for C73A was shown to be reduced to less than 1 h; a similar result was also obtained with other mutants showing reduced accumulation (mutants C51A, C51–54S, and H70-71L) (data not shown). These results clearly indicate that the reduced accumulation levels observed with some σ3 mutants result from decreased stability of the mutant proteins.

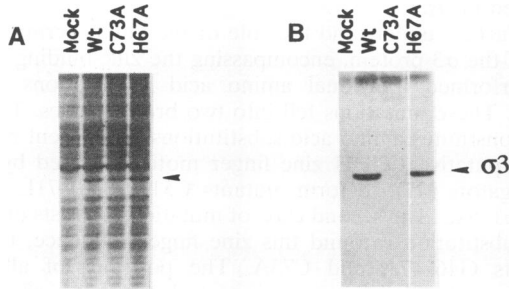

FIG. 2. Expression and accumulation of various σ3 mutants. COS cells were transfected with 10 μg of plasmid expression vector without inserts (mock) or expression vector encoding either wild-type σ3 (Wt) or the mutants as indicated. (A) Proteins were analyzed by metabolic radiolabeling, as described in the text. (B) Alternatively, proteins were transferred onto nitrocellulose filters and detected by immunoblotting with rabbit anti-reovirus antiserum and goat anti-rabbit immunoglobulin G–alkaline phosphatase conjugate.

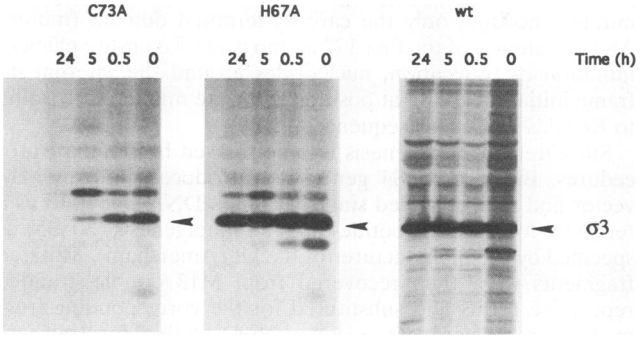

FIG. 3. Stability of mutated σ3 proteins. COS cells were transfected with 10 μg of plasmid expression vector encoding either wild-type σ3 (wt), mutant H67A, or mutant C73A. Proteins were pulse-labeled and then chased for the indicated period. Proteins were then recovered, immunoprecipitated, and analyzed by SDS-PAGE and autoradiography as described in the text. The position of the σ3 protein is indicated.

There is thus a possible correlation between amino acid substitution in the predicted zinc finger motif and decreased stability of the σ3 protein. However, substitution of histidine 67 in the predicted motif had no effect on stability. In contrast, substitution of cysteine 73 affected the stability even though it is not part of the predicted zinc finger sequence. We thus examined the participation of histidine 67 and cysteine 73 in the zinc-binding motif. The motif proposed by previous investigators (27) as the most probable zinc-binding site in σ3 was of the form Cys-X_2-Cys-X_{12}-His-X_3-His and corresponds to the classic zinc finger originally observed in TFIIIA (13) and also found in many DNA-binding proteins (2, 6). However, other zinc-binding motifs were later found to exist and consisted of various spatial arrangements of cysteine and histidine residues (2, 6). Among these is the so-called CCHC box, observed in some retroviral nucleocapsid proteins, which has been proposed to direct the specific packaging of viral RNA (4, 12). A close examination of the predicted amino acid sequences of all three serotypes of the reovirus σ3 protein shows, in addition to the putative CCHH zinc finger, another motif resembling the CCHC box. In this proposed Cys-X_2-Cys-X_{15}-His-X_3-Cys motif, compared with the previously suggested zinc finger, the first two cysteines will be the same while histidine 70 will probably substitute for histidine 71; the most important difference will be replacement of histidine 67 by cysteine 73 as the fourth amino acid involved in zinc atom tetracoordination.

To confirm this hypothesis concerning the nature of the zinc-binding motif, we further investigated the mutant proteins to establish the respective roles of cysteine 73 and histidine 67 in zinc binding. To directly examine the zinc-binding ability of each mutant, we used an assay based on a modification of chelate affinity chromatography (5, 26). In this assay, the agarose gel matrix contains zinc ions attached via biscarboxymethyl amino groups so that the zinc ions are able to adsorb metalloproteins (zinc chelate affinity adsorbent; Boehringer Mannheim). Transfected COS cells were subjected to metabolic radiolabeling with [^{35}S]methionine for 1 h at 42 h following transfection. Protein samples were obtained by lysis of the radiolabeled cells in column buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl) containing 1% Nonidet P-40. These samples were applied to the affinity column for 3 h; the column was then washed extensively with column buffer containing 0.1% bovine serum albumin, and bound proteins were eluted with 0.2 M EDTA (pH 6.0). The eluted proteins were analyzed by SDS-PAGE followed by densitometric analysis with a Phosphorimager (Molecular Dynamics). As shown in Fig. 4, mutant H67A bound to the zinc column whereas mutant C73A had lost this property. These results indicate clearly that cysteine 73 is involved in zinc attachment whereas histidine 67 is not. The other mutant which exhibited stability similar to the wild-type σ3 (G46–47A) was also found to bind to the zinc affinity column, whereas σ3 mutants with low stability (C51–54S, C51A, and H70–71L) did not bind (data not shown).

From these results, we conclude that the reovirus σ3 protein has a CCHC zinc-binding motif. More interestingly, the stability of σ3 correlates with its ability to bind a zinc atom. This observation also has important consequences for future mutagenesis studies. A similar correlation between stability and zinc binding has been well documented for the simian virus 40 small-t antigen (15, 21, 33) and suggested for at least one other viral protein, the human papillomavirus E7 protein (34).

The reason for the instability of the zinc-binding motif mutants in COS cells is not known. This might be explained if tetracoordination of the zinc atom is required to achieve a proper folding of the polypeptide chain. In the absence of zinc, the protein will exhibit an altered conformation leading to reduced stability, possibly through degradation by cellular proteases. In fact, it has been previously reported that zinc-binding domains may be required by some proteins to maintain proper conformation (23). Zinc-binding domains may also be involved in protein-protein interactions (see, e.g., references 3, 7, 8, and 23). Additional work is required to determine if the loss of zinc-binding capability can alter the interaction of σ3 with viral or cellular proteins. Interestingly, preliminary results suggest that the unstable mutants also exhibit an altered intracellular localization. This might reflect a difference in the interaction of σ3 with some cellular component.

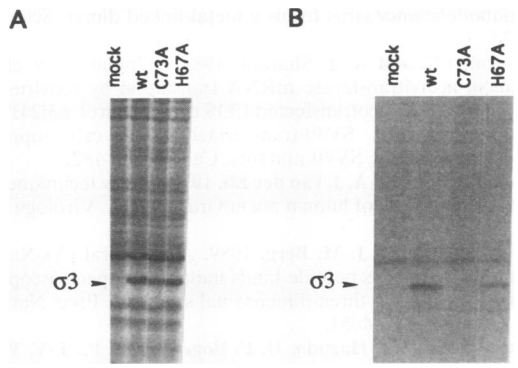

FIG. 4. Zinc-binding activity of mutated σ3 proteins. COS cells were transfected with 10 μg of plasmid expression vector without inserts (mock) or expression vector encoding either wild-type σ3 (wt), mutant H67A, or mutant C73A. Transfected and labeled COS cells were lysed and proteins were analyzed by zinc chelate affinity chromatography as described in the text. Proteins were resolved by SDS-PAGE, and dried gels were analyzed after overnight exposure in the Phosphorimager. (A) Aliquot of the starting material loaded onto the column. (B) Proteins retained onto the zinc column and eluted with 0.2 M EDTA.

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