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# NOTES

## The Rubella Virus RNA Binding Activity of Human Calreticulin Is Localized to the N-Terminal Domain

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**The rubella virus RNA 3′ *cis*-acting element, which is essential for viral negative-strand RNA synthesis, is specifically bound by autophosphorylated calreticulin. Autophosphorylation in recombinant human calreticulin occurs on serine and threonine residues. The RNA-binding and autophosphorylation activities were localized to the N-terminal 180 amino acids. Furthermore, N-terminal deletions revealed that the RNA-binding activity of calreticulin is abrogated upon deletion of the first 10 residues, whereas the autophosphorylation activity resides between amino acids 60 and 180. These results indicate that both the rubella virus RNA-binding and autophosphorylation activities of calreticulin are present in the N-terminal domain.**

Rubella virus (RV), the sole member of the *Rubivirus* genus in the family *Togaviridae*, possesses a positive-stranded RNA (9,757 nucleotides) genome (7). The 5′- and 3′-terminal RV RNA sequences contain stem-loop (SL) structures (7) implicated in RV RNA translation (15) and in replication (14). We have shown that an inverted GC-rich repeat sequence located near the 3′ end of the RV genomic RNA SL structure is necessary for initiation of negative-strand RNA synthesis (14). Specific high-affinity binding of cytosolic proteins from African green monkey kidney cells (Vero 76) to the RV 3′ SL structure was observed by UV-induced covalent cross-linking, and binding activity was dependent on the phosphorylation status (13). Furthermore, an increase in the affinity of host protein binding to RV RNA was observed following RV infection, coinciding temporally with the appearance of negative-strand RNA synthesis (13). Recently, we have purified the RV RNA-binding protein and identified it as the simian homolog of human calreticulin (16). Both in vivo and in vitro phosphorylated forms of calreticulin bind to the RV RNA 3′ SL (16).

Calreticulin was first identified in skeletal muscle sarcoplasmic reticulum (12). Recently, it has been shown that calreticulin modulates the expression of hormonally regulated genes and has also been localized in other cellular organelles, supporting the idea that it functions outside the endoplasmic reticulum (2, 5, 6). Three putative structural domains were identified in calreticulin: a globular N-terminal domain, a proline-rich P domain, and an acidic C-terminal tail domain (12). High- and low-affinity binding of $Ca^{2+}$ to calreticulin is restricted to the P and C domains, respectively (12). The globular N-terminal domain was shown to have affinity for the cytoplasmic domain of the alpha subunit of integrins and for a family of steroid receptors (2, 6, 12).

In the present study, we have identified the region of calreticulin that is autophosphorylated and interacts with RV RNA.

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**Serine and threonine residues are phosphorylated upon in vitro autophosphorylation of recombinant calreticulin.** It was shown that human calreticulin expressed in *Escherichia coli* as a fusion protein with maltose-binding protein (MBP-Cal) retains the capacity to bind to the RV 3′ positive-strand SL RNA upon in vitro phosphorylation, similar to the phosphorylated form of calreticulin purified from Vero 76 cell lysates (16). In order to identify the nature of the amino acids that are autophosphorylated in vitro, MBP-Cal was subjected to radiolabeled phosphoamino acid analysis by the method of Cooper et al. (4). Radiolabeled residues that comigrated with standard phosphoserine and phosphothreonine were identified by two-dimensional thin-layer electrophoresis as the phosphorylated residues (Fig. 1).

**Construction of MBP-Cal and derivative fusion proteins.** In order to define the RNA-binding and autophosphorylation domains of calreticulin, human calreticulin, its three individual domains (N, P, and C), and N-terminal deletion proteins fused to MBP were expressed in *E. coli*. A schematic representation of the constructs is shown in Fig. 2. Briefly, the ends of the coding sequence for mature human calreticulin, amino acids 1 to 417 (10), were modified by PCR to obtain 5′ *Eco*RI and 3′ *Hin*dIII restriction endonuclease sites. The DNA fragment obtained by PCR was digested with appropriate restriction enzymes and ligated into an expression vector, pMAL-c2 (New England Biolabs), at the *Eco*RI and *Hin*dIII sites as described before, in frame with MBP (16). By homology with rabbit calreticulin (12), the coding sequences of the N domain (residues 1 to 180, MBP-Cal-N), P domain (residues 181 to 289, MBP-Cal-P), and C domain (residues 290 to 417, MBP-Cal-C) of human calreticulin were individually amplified by PCR and cloned into pMAL-c2 at the *Eco*RI and *Hin*dIII sites. N-terminal deletions of the first 10, 19, 37, and 60 amino acids of mature calreticulin were generated by using 5′ and 3′ PCR primers to amplify the coding sequence corresponding to amino acids 11 to 417, 20 to 417, 38 to 417, and 61 to 417, respectively. The primers were designed to have 5′ *Eco*RI and 3′ *Hin*dIII sites so that the PCR products could be cloned into the pMAL-c2 vector as described above. The deletion constructs for the corresponding amino acids were designated

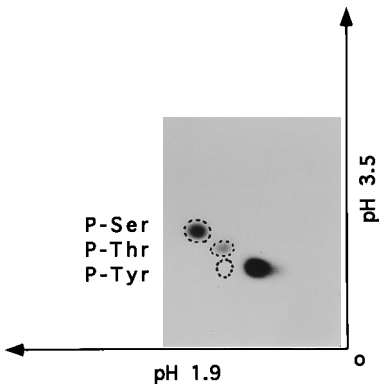


FIG. 1. Phosphoamino acid analysis of autophosphorylated MBP-Cal: representative two-dimensional electrophoretic profile of labeled recombinant calreticulin after acid hydrolysis. The sample origin is indicated (O). Dotted circles represent, from top to bottom, the mobility patterns of standard phosphoamino acids serine, threonine, and tyrosine, respectively. The autoradiographic spots, from top to bottom, comigrated with the standards representing phosphoserine and phosphothreonine, respectively. The spot on the right represents a product of incomplete hydrolysis.

MBP-Δ10Cal, MBP-Δ19Cal, MBP-Δ37Cal, and MBP-Δ60Cal, respectively (Fig. 2). The proteins were purified from *E. coli* according to the New England Biolabs protocol and stored in 10 mM Tris-Cl, pH 7.4. In vitro phosphorylation of the fusion proteins was carried out in the presence of either [$\gamma$-$^{32}$P]ATP (for the autophosphorylation assay) or unlabeled ATP (for RNA-protein binding assays). Autophosphorylation reactions and RNA-protein interactions by the RNA gel shift method were performed as described before (13, 16). Radiolabeled ([$\alpha$-$^{32}$P]CTP) RV 3′ positive-strand SL RNA (16) was transcribed from a T7 promoter as described before (13).

**Identification of RV RNA-binding and autophosphorylation regions of calreticulin.** Figure 3A shows Coomassie blue staining of the proteins expressed and demonstrates the integrity of the MBP, MBP-Cal fusion proteins, and individual domains fused to MBP. The migration of the proteins corresponds to the expected molecular weights. Autophosphorylation of recombinant proteins showed that both MBP-Cal (Fig. 3B, lane 2) and MBP-Cal-N (Fig. 3B, lane 3) were autophosphorylated.

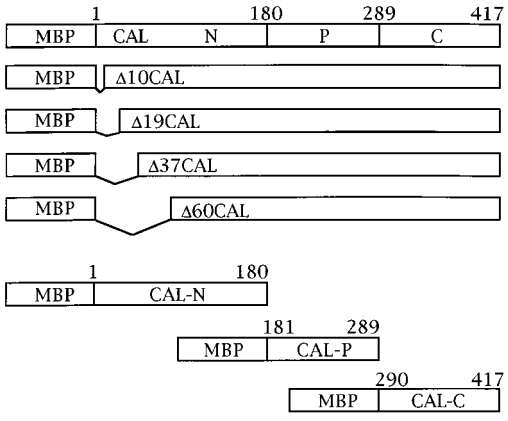


FIG. 2. Schematic representation of full-length, truncated portions, and individual domains of human calreticulin expressed in *E. coli* as fusion proteins with MBP. Fusion proteins are represented as boxes. Deletions in the N terminus of calreticulin are shown. Numbers above each box denote the amino acid numbers, taken from mature calreticulin (10).

Neither MBP-Cal-P (Fig. 3B, lane 4) nor MBP-Cal-C (Fig. 3B, lane 5) was autophosphorylated. MBP alone also did not undergo autophosphorylation (Fig. 3B, lane 1). Since it is not known which serine and threonine residues in calreticulin are phosphorylated, we cannot rule out at this time whether the lack of phosphorus labeling of the P and C domains of calreticulin is due to the absence of the N domain. The N, P, and C domains were subjected to the autophosphorylation reaction with nonradioactive ATP and then used in the RV RNA-binding assay. Only the phosphorylated MBP-Cal (Fig. 3C, lane 2) and MBP-Cal-N (Fig. 3C, lane 3) bound to the RV RNA. MBP, MBP-Cal-P, and MBP-Cal-C showed no RNA-binding activity (Fig. 3C, lanes 1, 4, and 5). These data demonstrate that the RV RNA-binding activity of calreticulin is located within the N domain and depends on its phosphorylation status.

In order to further localize the RNA binding and phosphorylation within the N domain, MBP-Cal proteins bearing deletions of 10, 19, 37, and 60 amino acids from the N terminus were expressed. Both the truncated and MBP-Cal fusion proteins were subjected to an in vitro autophosphorylation reaction with [$\gamma$-$^{32}$P]ATP (Fig. 4A). MBP alone was not labeled (Fig. 4A, lane 1), whereas both MBP-Cal and all of the truncated calreticulin derivatives were labeled (Fig. 4A, lanes 2 to 6), suggesting that the autophosphorylation activity of calreticulin was not affected by deletion of the first 60 residues and that the residues between 60 and 180 of the N domain are essential for the autokinase activity of calreticulin. The autophosphorylated proteins were then subjected to the RV 3′ positive-strand SL RNA gel shift assay. Only phosphorylated MBP-Cal bound to the RV RNA (Fig. 4B, lane 2). Neither MBP-Δ10Cal nor MBP-Δ19Cal bound to RV RNA (lanes 3 and 4). As observed previously, MBP also did not bind RV RNA (Fig. 4B, lane 1). Proteins with deletions of 37 and 60 residues at the N terminus of calreticulin (MBP-Δ37Cal and MBP-Δ60Cal, respectively) also showed no RNA-binding activity (data not shown). These results suggest that the N-terminal 10 amino acids of calreticulin are necessary for its RNA-binding activity.

In the present study, we showed that the novel RNA-binding and autophosphorylation activities of calreticulin are present in the putative N-terminal domain. The N-terminal 10 amino acids of calreticulin contribute essential functions to RNA-binding activity, whereas a region between amino acids 60 and 180 of the N domain contribute to autophosphorylation activity. Our results support the idea that calreticulin acquires RNA-binding activity in vitro only when the serine and threonine residues are autophosphorylated. Many RNA-binding proteins which show conserved ribonucleoprotein binding motifs have been identified to date (1). However, when we searched for such motifs in calreticulin, none were present in the calreticulin protein. Lack of a typical RNA-binding motif is not unique to calreticulin, since other RNA-binding proteins, including glyceraldehyde-3-phosphate dehydrogenase and iron response element-binding protein, do not possess such motifs either (9, 17). The RNA-binding activities of these two proteins are regulated by the presence or absence of their cofactors, $NAD^+$ and iron-sulfur cluster, respectively (9, 17). Since phosphorylation of calreticulin is crucial for RV RNA binding, we believe that regulation of phosphorylation upon RV infection allows calreticulin to become a high-affinity RV RNA-binding protein (13). These observations lead us to speculate that there are several ways by which proteins acquire RNA-binding activities: (i) the proteins possess RNA recognition motifs; (ii) the proteins have different sequence motifs yet to be identified; or (iii) posttranslational modifications expose

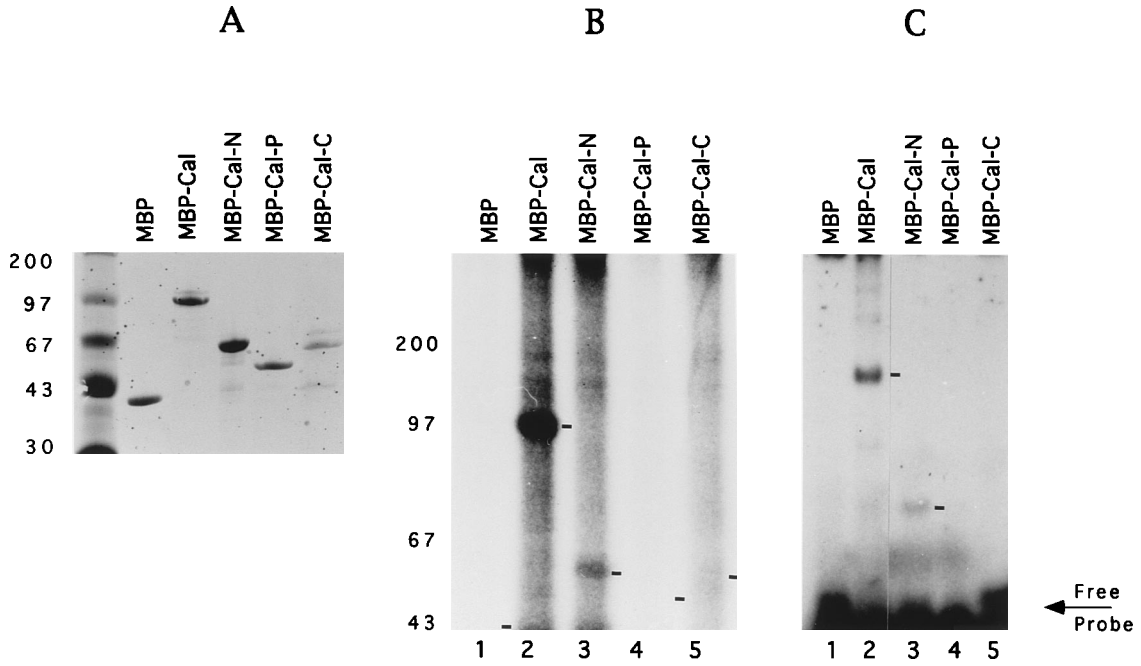


FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, autophosphorylation, and RNA-binding activity of recombinant calreticulin and its putative N, P, and C domains. (A) SDS-PAGE of fusion proteins stained with Coomassie blue. Samples are identified above each lane. The positions of protein molecular size markers are indicated on the left (in kilodaltons). (B) SDS-PAGE profile of autophosphorylated proteins in the presence of [$\gamma$-$^{32}$P]ATP. Lanes: 1, MBP; 2, MBP-Cal; 3, MBP-Cal-N; 4, MBP-Cal-P; 5, MBP-Cal-C. Numbers on the left represent molecular sizes of marker proteins (in kilodaltons). (C) RNA gel retardation assay of labeled RV 3′ positive-strand SL RNA with phosphorylated proteins in the presence of unlabeled ATP. Lanes: 1, MBP; 2, MBP-Cal; 3, MBP-Cal-N; 4, MBP-Cal-P; 5, MBP-Cal-C. The black bars to the right of each lane mark the positions of the protein (B) or the protein-RNA complex (C).

appropriate amino acids competent to bind RNA. By virtue of its lack of a consensus RNA recognition motif and dependence on phosphorylation (16), calreticulin can be grouped into the third category of proteins.

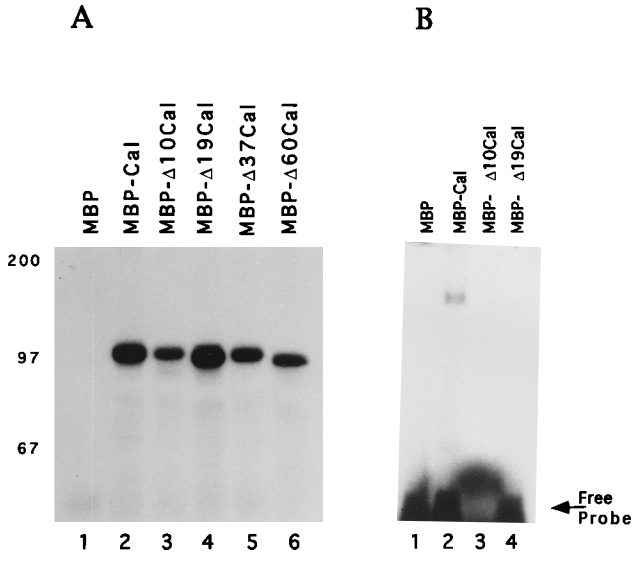


FIG. 4. Autophosphorylation and RNA-binding activity of recombinant calreticulin and its N-terminal deletion proteins. (A) SDS-PAGE profile of autophosphorylated proteins in the presence of [$\gamma$-$^{32}$P]ATP. Lanes: 1, MBP; 2, MBP-Cal; 3, MBP-Δ10Cal; 4, MBP-Δ19Cal; 5, MBP-Δ37Cal; 6, MBP-Δ60Cal. Numbers on the left represent the molecular sizes of marker proteins (in kilodaltons). (B) RNA gel retardation assay of labeled RV 3′ positive-strand SL RNA with phosphorylated proteins in the presence of unlabeled ATP. Lanes: 1, MBP; 2, MBP-Cal; 3, MBP-Δ10Cal; 4, MBP-Δ19Cal. The position of free probe is indicated.

Calreticulin is a moderately abundant protein in Vero cells, but only a fraction of the protein is phosphorylated (16). Since the majority of calreticulin is known to be involved in $Ca^{2+}$ storage, only a minor portion (possibly catalytic amounts) of calreticulin may be involved in RV RNA binding (16). Perhaps the form of calreticulin that binds to RV RNA also associates with other cellular RNAs, although these RNAs have yet to be identified. Thus, it seems that the host cell (Vero 76) uses phosphorylation as a mechanism to create a pool of functionally diverse calreticulin protein. Post-translational modifications such as phosphorylation are known to have regulatory functions that affect the properties of a wide range of proteins (1, 3). For example, in human heterogeneous nuclear ribonucleoprotein A1, phosphorylation by cyclic AMP-dependent protein kinase A suppresses the capacity of protein A1 to promote strand annealing in vitro without altering its ability to bind to nucleic acid (3).

During RV infection, calreticulin is hyperphosphorylated, and its binding affinity for RV RNA increases temporally with the onset of negative-strand synthesis (13). This observed correlation between increased RV RNA-calreticulin interaction and the appearance of virus negative-strand synthesis could be interpreted in several ways. One possibility is that this interaction supports a productive infection of the virus by promoting viral RNA replication (14) or translation (15). Another possibility is that this interaction selectively targets viral RNA for degradation during host defense processes against RV infection. Alternatively, calreticulin could compartmentalize RV RNA in cellular organelles to avoid surveillance by the host immune system, leading to RV persistence. Several cellular proteins have been shown to bind regulatory elements of viral RNAs, but in a majority of the cases, the function of such interactions is not well understood (8, 11). However, only recently, the La autoantigen has been shown to modulate trans-

lation of poliovirus RNA (11) and human immunodeficiency virus type 1 mRNA (18).

The consequence of the interaction between calreticulin and RV RNA is speculative in nature. However, our results demonstrate that a host protein, calreticulin, whose major function is to regulate $Ca^{2+}$ storage, can also be activated to bind RNA by autophosphorylation. The RNA-binding and autokinase activities are present in a distinct domain of the calreticulin, separate from the $Ca^{2+}$-binding domains.

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