Ca^{2+}-dependent and Ca^{2+}-independent Calmodulin Binding Sites in Erythrocyte Protein 4.1

IMPLICATIONS FOR REGULATION OF PROTEIN 4.1 INTERACTIONS WITH TRANSMEMBRANE PROTEINS*

(Received for publication, March 25, 1999, and in revised form, October 1, 1999)

Wataru Nunomura‡§, Yuichi Takakuwa‡, Marilyn Parra§, John G. Conboy§, and Narla Mohandas§

From the ‡Department of Biochemistry, School of Medicine, Tokyo Women’s Medical University, Shinjuku, Tokyo 162-8666, Japan and §Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720

In vitro protein binding assays identified two distinct calmodulin (CaM) binding sites within the NH2-terminal 30-kDa domain of erythrocyte protein 4.1 (4.1R): a Ca^{2+}-dependent binding site (A^{266}KKLWKVCVEHITFFRL) and a Ca^{2+}-independent binding site (A^{181}KKLSMYGVDLHKAKDL). Synthetic peptides corresponding to these sequences bound CaM in vitro; conversely, deletion of these peptides from a 30-kDa construct reduced binding to CaM. Thus, 4.1R is a unique CaM-binding protein in that it has distinct Ca^{2+}-dependent and Ca^{2+}-independent high affinity CaM binding sites. CaM bound to 4.1R at a stoichiometry of 1:1 both in the presence and absence of Ca^{2+}, implying that one CaM molecule binds to two distinct sites in the same molecule of 4.1R. Interactions of 4.1R with membrane proteins such as band 3 is regulated by Ca^{2+} and CaM. While the intrinsic affinity of the 30-kDa domain for the cytoplasmic tail of erythrocyte membrane band 3 was not altered by elimination of one or both CaM binding sites, the ability of Ca^{2+}/CaM to down-regulate 4.1R-band 3 interaction was abrogated by such deletions. Thus, regulation of protein 4.1 binding to membrane proteins by Ca^{2+} and CaM requires binding of CaM to both Ca^{2+}-independent and Ca^{2+}-dependent sites in protein 4.1.

Calmodulin (CaM) is a highly conserved Ca^{2+}-binding protein that modulates the functional activities of different structural and transport proteins and the activities of many Ca^{2+}-dependent enzymes (for a review, see Refs. 1 and 2). In most cases, Ca^{2+} is essential for the initial binding of CaM to these proteins and for subsequent modulation of their function. However, a small subset of proteins including erythroid 4.1 (4.1R) bind CaM in a Ca^{2+}-independent manner but require Ca^{2+} for manifesting changes in function. Of particular relevance to the present study is the role played by CaM in modulating various protein-protein interactions in the erythrocyte membrane involving 4.1R.

CaM is present in human erythrocytes at micromolar concentration (3–5). In these cells, CaM binds to Ca^{2+}-ATPase (6, 7) with a dissociation constant on the order of 10 nM (8), while it binds to membrane skeletal proteins, 4.1R, and adducin with a dissociation constant on the order of 0.1–0.2 μM (3, 9–12). Through its interaction with Ca^{2+}-ATPase, CaM enables the erythrocyte to maintain a submicromolar concentration of Ca^{2+}. Since only 5% of the CaM in erythrocyte is involved in interaction with Ca^{2+}-ATPase (13), 95% of CaM is available for interaction with other membrane proteins and modulation of their functional interactions. In fact, recent evidence suggests that Ca^{2+}/CaM reduces the affinities of the spectrin-actin-adducin interaction (9, 14) and of the spectrin-actin-4.1 interaction (11, 15). Ca^{2+}/CaM also decreases the affinity of interaction between 4.1R and band 3 and between 4.1R and glycophorin C (16). By modulating the affinities of these different protein-protein interactions, Ca^{2+}/CaM can play a significant role in regulating the function of the erythrocyte membrane (10, 11, 15, 16).

Biochemical and biophysical studies have facilitated construction of a structural and functional map of the 4.1R molecule (for a review, see Ref. 17). Four major structural domains of 4.1R with apparent molecular masses of 30, 16, 10, and 22–24 kDa were identified. 4.1R interacts with spectrin and actin through its 10-kDa domain and with integral membrane proteins glycophorin C and band 3 through its 30-kDa domain (reviewed in Ref. 17). CaM has previously been shown to bind to the 30-kDa domain of 4.1R at a molar ratio of 1:1 in a Ca^{2+}-independent manner (11). However, there is an absolute Ca^{2+} requirement for CaM-induced regulation of 4.1R interactions with other membrane proteins (15, 16). To understand the molecular basis for this Ca^{2+} requirement, we explored the possibility that the presence of both Ca^{2+}-dependent and Ca^{2+}-independent CaM binding sites in the 30-kDa domain may account for CaM regulation of protein-protein interactions involving protein 4.1R.

In the present study, we quantitated the affinity of interaction between CaM and 4.1R, its 30-kDa domain, and various synthetic peptides. CaM bound to the 30-kDa domain of 4.1R in Ca^{2+}-independent manner at a molar ratio of 1:1 with a dissociation constant on the order of 0.1 μM, consistent with previous findings (11). We identified two different CaM-binding sequence motifs in the 30-kDa domain of 4.1R: one encoded by exon 9 (AKKLSMYGVDLHKAKDL; peptide 9) and the other by exon 11 (AKKLWKVCVEHITFFRL; peptide 11) (18). These sequences share conserved sequence motifs with the CaM binding sequence of Ca^{2+}-ATPase (6). Importantly, while peptide 11...
bound CaM with high affinity in the absence of Ca$^{2+}$, binding of CaM to peptide 9 was Ca$^{2+}$-sensitive. Serine 185 in peptide 9 appeared to play a critical role in Ca$^{2+}$-dependent CaM binding, since replacement of this serine by tryptophan resulted in loss of Ca$^{2+}$-dependent binding of CaM to 4.1R. Ca$^{2+}$ decreases the affinity of band 3 binding to the 30-kDa domain in the presence of CaM. However, Ca$^{2+}$ had no effect on the affinity of the band 3 interaction with the 30-kDa domain when either the Ca$^{2+}$-independent or both the Ca$^{2+}$-dependent and -independent CaM binding sites were deleted. Thus, regulation of 4.1R binding to membrane proteins by Ca$^{2+}$ and CaM requires binding of CaM to both Ca$^{2+}$-dependent and Ca$^{2+}$-independent sites in 4.1R. Based on these findings, we propose that two distinct domains in 4.1R are responsible for CaM binding and that one of these domains is responsible for Ca$^{2+}$-sensitive regulation of 4.1-R interactions with membrane proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**

Phenyl-Sepharose 4B, CaM-Sepharose CL-4B, glutathione-Sepharose 4B, and Sephadex G-25 were purchased from Amersham Pharmacia Biotech. Fmoc-amino acids and Fmoc-amino acid-coupled resins were obtained from PerSeptive Biosystems, Co. Ltd. (Boston, MA). N’-Hydroxysuccinimide (NHS) and streptavidin were purchased from Pierce. IAsys™ cuvettes coated with biotin via the spacer sequence, SGSG, at the amino terminus of these peptides followed by biotinylation using succinimide ester (26).

**Preparation of Recombinant Proteins—**Recombinant 30-kDa domain of 4.1R (r30kDa), r30kDa from which sequences encoded by exons 5, 9, or both exons 9 and 11 had been deleted (ΔEx.5, ΔEx.9, and ΔEx.9/11, respectively; Fig. 1A), and a recombinant protein corresponding to the cytoplasmic domain of band 3 (27) were produced as glutathione S-transferase fusion proteins in Escherichia coli. cDNAs encoding these different sequences were amplified by polymerase chain reaction using specific primers, and the minigenes were constructed in modified pGEX-KG vector and sequenced (19). The fusion proteins were expressed as described previously (28, 29) and purified from bacteria lysates by affinity column chromatography using glutathione-Sepharose 4B. The purified fusion proteins were cleaved with thrombin. The replacement of Ser185 by Trp185, of Phe277-Phe278 by Ala277-Ala278, and of Trp268 and Phe277-Phe278 by Ala268 and Ala277, Ala278 in r30kDa in the pGEX-KG vector was performed using the QuikChange™ site-directed mutagenesis kit. r30kDa was further purified by affinity column chromatography using CaM-Sepharose CL-4B (16), while the other recombinant proteins were purified using glutathione-Sepharose 4B to remove cleaved glutathione S-transferase. The purity of the recombinant proteins was assessed by SDS-polyacrylamide gel electrophoresis (15% gel) analysis. The protein concentrations were determined using the following relationship: protein concentration (mg/ml) = 1.45 A$_{280}$ – 0.74 A$_{260}$.  

**Binding Assay by Resonant Mirror Detection—**Protein-protein interactions and protein-peptide interactions were studied using the resonant mirror detection method (30–32) of the IAsys™ system (Affinity Sensors, Cambridge, UK). To immobilize 30-kDa in 4.1R and CaM on the binding of different r30kDa proteins to transmembrane proteins, the recombinant cytoplasmic domain of band 3 was immobilized on the surface of the amineosilane cuvette according to the manufacturer’s instructions with slight modifications (32). All experimental procedures were carried out at 25 °C with constant stirring. In brief, an amineosilane cuvette was activated with 2 μL of bio(sulfosuccinimidyl)suberate. After extensive washing with distilled water, proteins suspended in phosphate-buffered saline (10 mM Na$_2$HPO$_4$/NaH$_2$PO$_4$, pH 7.4, containing 0.15 μM NaCl) at a concentration of 0.1 mg/ml were added to the cuvette and incubated for 30 min at 25 °C. After washing with phosphate-buffered saline, 2 mg/ml of bovine serum albumin in phosphate-buffered saline was added to the cuvette to reduce nonspecific binding. The biotinylated peptides were immobilized onto the cuvette surface through streptavidin. Cuvettes with immobilized bovine serum albumin alone or streptavadin alone were prepared to serve as negative controls for binding studies. To quantitate the effects of Ca$^{2+}$ and CaM on the binding of different r30kDa proteins to transmembrane proteins, the recombinant cytoplasmic domain of band 3 was immobilized on the surface of the amineosilane cuvette. r30kDa or its mutants (50 nM-1 μM) were preincubated with 5 μM CaM in buffer A (20 mM imidazole HCl, pH 7.2, containing 0.1 M NaCl) and either 0.1 mM EGTA or 1.1 mM CaCl$_2$ and 1.0 mM EGTA at 25 °C for 30 min. All binding assays were conducted in buffer A and 0.1 mM EGTA or 1.1 mM CaCl$_2$ and 1.0 mM EGTA at 25 °C for 30 min. The dissociation constant K$_{Diss}$ was determined from the binding assay as described previously (33). In brief, the time-dependent changes in arc seconds provided information concerning real-time changes in surface concentration of protein complexes formed on the sensor surface (30–32). The resulting binding curve (arc seconds versus time; see Fig. 2) was ana-
lyzed using the software package FASTfitTM (Affinity Sensors, Cambridge, UK). The dissociation constant from this form of kinetic analysis (termed \( K(D)_{kin} \)) is then calculated as follows: \( K(D)_{kin} = \frac{k_d}{k_a} \), where \( k_a \) is the association rate constant and \( k_d \) is the dissociation rate constant.

The dissociation constant by Scatchard plot analysis (termed \( K(D)_{Scat} \)) was also derived from the binding data (33). In the present study, the \( K(D)_{Scat} \) derived under all experimental conditions closely matched the corresponding \( K(D)_{kin} \) values calculated.

The stoichiometry of 4.1R binding to CaM in the presence and absence of Ca\(^{2+}\) was determined using the IAsys™ system. Maximal binding (\( B_{max} \)) of 4.1R represented as arc seconds was obtained from the Scatchard plot as described previously (33). The amount of immobilized CaM on the aminosilane cuvette was determined as the difference of arc seconds between bis(sulfosuccinimidyl)suberate and CaM under equilibrium conditions. The stoichiometry of 4.1R binding to CaM was calculated according to the following equation described in the Method Guide of the IAsys™ system: Stoichiometry of 4.1R:CaM = \( \frac{B_{max} \text{ of } 4.1R}{80,000} \) : \( \frac{\text{amount of immobilized CaM on aminosilane cuvette}}{18,000} \), where 80,000 and 18,000 are apparent molecular weights of 4.1R (18) and CaM (1, 2), respectively.

The cuvettes were reused after cleaning with HCl. Original binding curves could be replicated after HCl washes, implying that the washing procedure used did not denature the bound ligands.

RESULTS

30-kDa Domain of 4.1R Binds CaM—The ability of CaM to bind to native 4.1R and to its four major structural domains (30-, 16-, 10-, and 22/24-kDa domains) was determined using the IAsys™ system. All binding studies were performed at physiologic ionic strength. Ca\(^{2+}\) dependence of binding was evaluated at defined concentrations of Ca\(^{2+}\). Analysis of binding response curves (Fig. 2) obtained at varying concentrations of 4.1R in the presence of EGTA (Fig. 2A) provided \( k_a \) and \( k_d \) of 6.1 \( \times 10^4 \) M\(^{-1}\) s\(^{-1}\) and 2.0 \( \times 10^{-2} \) s\(^{-1}\), respectively. From these measured values of \( k_a \) and \( k_d \), a \( K(D)_{kin} \) value of 0.33 \( \mu \)M was derived for Ca\(^{2+}\)-independent CaM binding to 4.1R. Ca\(^{2+}\) had little effect on the affinity of 4.1R binding to CaM. The stoichiometry of 4.1R binding to CaM was calculated according to the following equation described in the Method Guide of the IAsys™ system: Stoichiometry of 4.1R:CaM = \( \frac{B_{max} \text{ of } 4.1R}{80,000} \) : \( \frac{\text{amount of immobilized CaM on aminosilane cuvette}}{18,000} \), where 80,000 and 18,000 are apparent molecular weights of 4.1R (18) and CaM (1, 2), respectively.

![Figure 2](http://www.jbc.org/)

**FIG. 2.** Tracings from the IAsys™ system to quantitate the interactions between CaM and 4.1R (A), r30kDa (B), and a mixture of 16-, 10-, and 22/24-kDa domains of 4.1R (C). The overlay plots show specific association and dissociation phases generated by increasing concentrations of 4.1R (A) and r30kDa (B). In contrast, no specific association was noted between CaM and either a mixture of 16-, 10-, and 22/24-kDa domains of 4.1R or heat-denatured r30kDa (C).

![Figure 3](http://www.jbc.org/)

**FIG. 3.** Scatchard plot analysis of 4.1R binding to CaM. A, concentration dependence of 4.1R binding to CaM is shown. 4.1R (up to 0.8 \( \mu \)M) was incubated with the CaM immobilized on the aminosilane cuvette in the presence (○) and absence (□) of Ca\(^{2+}\). The amounts of the 4.1R and CaM complex under equilibrium conditions are represented as Response (arc seconds). B, Scatchard analysis of binding data from A.
Calmodulin Binding Sites in Protein 4.1

Table I

| Protein | Mutation | Condition | \( K_{D_{\text{kin}}} \) μM |
|---------|----------|-----------|------------------------|
| 4.1R    | Wild type | EGTA      | 0.33 ± 0.02            |
| r30kDa  | Wild type | EGTA      | 0.35 ± 0.03            |
|         | +Ca²⁺    | EGTA      | 0.13 ± 0.01            |
|         | Ex.9/11  | EGTA      | 3.0 ± 0.26             |
|         | +Ca²⁺    | EGTA      | 0.09 ± 0.02            |
|         | Ex.9/11  | No binding| 0                      |
|         | +Ca²⁺    | No binding| 0                      |

Table II

| Peptide | Mutation | Condition | \( K_{D_{\text{kin}}} \) μM |
|---------|----------|-----------|------------------------|
| 9       | Wild type | EGTA      | 40 ± 5.30              |
|         | + peptide 11 | EGTA  | 5.6 ± 0.55          |
|         | Serⁱ⁸⁵ → Trp¹⁸⁵ | EGTA | 0.34 ± 0.08        |
|         | Trp⁶⁶⁵ → Ser⁶⁶⁵ | EGTA | 8.5 ± 0.51       |
|         | +Ca²⁺    | EGTA      | 5.6 ± 0.10            |
| 11      | Wild type | EGTA      | 0.54 ± 0.01            |
|         | Trp⁶⁶⁵ → Ser⁶⁵ | EGTA | 6.7 ± 0.02          |
|         | +Ca²⁺    | EGTA      | 2.8 ± 0.10            |
|         | Trp⁶⁷⁵,Lys⁶⁹⁵ → Ser⁶⁶⁵,Leu⁶⁹⁵ | EGTA | 21 ± 0.24 |
|         | +Ca²⁺    | EGTA      | 1.1 ± 0.01            |

Table I at a molar ratio of 1:1. Again, Ca²⁺ had little effect on the affinity or stoichiometry of CaM binding to the 30-kDa domain. In contrast, no binding signal could be detected for interaction between CaM and 16-, 10-, or 22/24-kDa domains of 4.1R (Fig. 2C). Thus, CaM binds to the 30-kDa domain of 4.1R in a Ca²⁺-independent fashion, consistent with earlier reports (11, 16). However, these results do not rule out the presence of additional Ca²⁺-dependent binding sites, of equal or lower affinity, whose existence could be masked by the high affinity Ca²⁺-independent binding site.

Calmodulin Binding Sites in the 30-kDa Domain of 4.1R—To identify CaM binding sites in the 30-kDa domain of 4.1R, we quantitated CaM binding to r30kDa from which sequences encoded either by exon 11 (Ex.11) or both exons 9 and 11 (Ex.9/11) had been deleted. As noted before, CaM binds wild type r30kDa with a \( K_{D_{\text{kin}}} \) value on the order of 0.1 μM either in the presence or absence of Ca²⁺. Deletion of sequences encoded by both exons 9 and 11 (Ex.9/11) completely abolished the binding of the mutant r30kDa peptide to CaM either in the presence or absence of Ca²⁺ (Table I). This finding implies that CaM binding domain sequences are encoded by these two exons.

Interestingly, the mutant r30kDa ΔEx.11 polypeptide exhibited Ca²⁺-sensitive CaM binding with a \( K_{D_{\text{kin}}} \) value of 30 μM in the absence of Ca²⁺ and a \( K_{D_{\text{kin}}} \) value of 5.1 μM in the presence of Ca²⁺ (Table I). These data imply that sequences encoded by exon 9 exhibit Ca²⁺-sensitive CaM binding. It should be noted that the affinity of the interaction of this mutant protein with CaM was markedly decreased compared with the wild type protein, suggesting that sequences encoded by exon 11 may constitute the high affinity binding site in 30-kDa domain for CaM. We were unable to study CaM binding to ΔEx.9 r30kDa, since this recombinant protein could not be obtained in a soluble form. Deletion of sequences encoded by exon 5 (Ex.5) from the r30kDa polypeptide had no effect on CaM binding in the presence or absence of Ca²⁺ (data not shown). Taken together, these data imply that two distinct CaM binding sites encoded by exons 9 and 11 exist in 4.1R.

Identification of Sequence Motifs in the 30-kDa Domain of 4.1R That Bind CaM—We identified the putative CaM binding sequence motif, AKKXXXXXXY/VHXXXY (where the XXX indicates that there may be 2 or 3 unknown residues) in the sequences encoded by both exons 9 and 11. The sequence encoded by exon 9 is AKKLSMYGVDLHKKAKDL (peptide 9), while the one encoded by exon 11 is AKKLWVKCVCVHEHHTF-FRL (peptide 11) (Fig. 1B). To test directly whether these sequences can bind CaM, the corresponding synthetic peptides were made, and their binding to CaM was assayed (Table II). CaM bound to peptide 11 with a \( K_{D_{\text{kin}}} \) value of 0.54 μM both in the presence and absence of Ca²⁺. CaM binding to peptide 9 was highly sensitive to Ca²⁺. CaM binding to peptide 9 with a \( K_{D_{\text{kin}}} \) value of 1.7 μM in the presence of Ca²⁺ but with a much higher \( K_{D_{\text{kin}}} \) value of 40 μM in the absence of Ca²⁺. Peptide 9-a (Δ³⁸⁵-KKLSMYGV) also showed Ca²⁺-sensitive CaM binding, while binding of peptide 9-b (D³⁹⁵LHKKAKDL) to CaM was not Ca²⁺-sensitive (data not shown). It is noteworthy that there was an absolute requirement for Ca²⁺ for interaction of CaM with peptide 9-a. These data suggest that the Ca²⁺-sensitive CaM binding site in peptide 9 requires sequences in peptide 9-a.

The binding assays thus revealed two distinct CaM binding sites in 4.1R: a Ca²⁺-independent site in peptide 11 and a Ca²⁺-dependent site in peptide 9. Further analysis of the data showed that although both peptide 9 and peptide 11 bind CaM in the absence of Ca²⁺, the affinity of Ca²⁺-independent binding of CaM to peptide 11 was almost 80-fold higher than that of CaM binding to peptide 9. This difference was mainly due to differences in the \( K_{D_{\text{kin}}} \) of CaM interactions with these two peptides; \( K_{D_{\text{kin}}} \) values of 6.5 × 10⁻³ M⁻¹ s⁻¹ for peptide 9 and 5.4 × 10⁻⁴ M⁻¹ s⁻¹ for peptide 11). Furthermore, while Ca²⁺ had no effect on the interaction of CaM with peptide 11, it had a marked effect on the interaction of CaM with peptide 9. In fact, Ca²⁺ increased the affinity of the CaM-peptide 9 interaction 25-fold to a value very close to that of the interaction between CaM and peptide 11 in either the presence or absence of Ca²⁺. This large increase in affinity induced by Caco²⁺ is in large part due to its effect on the \( K_{D_{\text{kin}}} \) of the CaM-peptide 9 interaction. We suggest that this Ca²⁺-induced increase in \( K_{D_{\text{kin}}} \) values is most likely due to Ca²⁺-induced change in the conformation of CaM.

In effect, these binding data suggest that CaM may bind preferentially to peptide 11 in the absence of Ca²⁺ and that increased Ca²⁺ may stimulate binding to a second site in peptide 9. To further validate a role for Ca²⁺ in modulating the interaction of CaM with peptide 9, we measured the ability of a preformed CaM-peptide 11 complex to bind peptide 9 in the presence and absence of Ca²⁺ (Table II). As with native CaM, the CaM-peptide 11 complex bound peptide 9 (Table II) as well as peptide 9-a (data not shown) in a Ca²⁺-dependent manner.
Binding to peptide 9-b once again was not Ca\(^{2+}\)-sensitive (data not shown). The affinity between the CaM-peptide 11 complex and peptide 9 was an order of magnitude higher than the affinity between native CaM and peptide 9 (Table II), suggesting potential cooperativity between peptide 9 and peptide 11 sequences in modulating the binding of the 30-kDa domain to CaM.

CaM bound to peptides 9 and 11 at physiologically relevant ionic strength (0.15 mM NaCl). However, CaM began to dissociate from these peptides at higher ionic strengths (0.2 M NaCl and greater) with complete dissociation observed at 0.6 M NaCl (data not shown). CaM did not bind to various synthetic peptides representing sequences surrounding the CaM binding sequence motifs identified in the 30-kDa domain. In particular, CaM did not bind to peptides encoding the following sequence motifs: E\(^{190}\)GVDII (sequence downstream of peptide 9), T\(^{281}\)STDTPK (sequence downstream of peptide 11), or I\(^{244}\)RPGEIQVESTIFKLPYRA (sequence upstream of peptide 11) either in the presence or absence of Ca\(^{2+}\) (data not shown). In addition, CaM did not bind to bovine serum albumin or streptavidin in our assay system (data not shown).

**Serine\(^{185}\) in Peptide 9 Is Critical for Ca\(^{2+}\)-dependent Binding to CaM—**To determine the molecular basis for the differences in Ca\(^{2+}\) sensitivity for CaM binding to peptides 9 and 11, we performed binding studies using peptides 9 and 11 with defined mutations. Although the first four amino acids are identical in both peptides (A\(^{181}\)KKL in peptide 9 and A\(^{264}\)KKL in peptide 11), the fifth amino acid residue is different (Ser\(^{185}\) in peptide 9 and Trp\(^{268}\) in peptide 11) (Fig. 1). Replacing this Ser\(^{185}\) residue in peptide 9 with Trp in effect converted the Ca\(^{2+}\)-dependent binding site into a Ca\(^{2+}\)-independent site, mainly by enhancing CaM binding in the absence of Ca\(^{2+}\) (Table II). Similar Ca\(^{2+}\)-independent binding was noted using the r30kDa domain in which Ser\(^{185}\) was mutated to Trp\(^{185}\) (data not shown). More importantly, it was possible to impart Ca\(^{2+}\)-sensitive binding to peptide 11 by replacing residues Trp\(^{268}\), Lys\(^{269}\) in peptide 11 with Ser\(^{268}\)-Leu\(^{269}\). This effect is due to a decreased \(k_\text{diss} \) of the Ser\(^{268}\)-Leu\(^{269}\) mutant for CaM in the absence of Ca\(^{2+}\); \(k_\text{diss} \) values for 5.4 \(\times 10^4\) M\(^{-1}\) s\(^{-1}\) for wild type peptide and 5.6 \(\times 10^3\) M\(^{-1}\) s\(^{-1}\) for mutant peptide). Interestingly, replacing just the tryptophan residue in peptide 11 with the serine residue in the CaM binding motif of 4.1R plays a critical role in imparting Ca\(^{2+}\)-sensitive binding.

**Aromatic Amino Acids in Peptide 11 Are Critical for Ca\(^{2+}\)-independent Binding to CaM—**It has been previously documented that tryptophan and phenylalanine play a critical role in Ca\(^{2+}\)-dependent binding of CaM (1). In order to determine if aromatic amino acids also play an important role in Ca\(^{2+}\)-independent binding of CaM to 4.1R, we measured its binding to peptide 11 in which different amino acid substitutions were made. Replacement of Trp\(^{268}\) by alanine markedly decreased the affinity for CaM (Table III). Whereas the wild type peptide bound with a \(K_{D,\text{diss}} \) of \(\sim 0.5\) \(\mu\)M, the W268A mutant had an increased \(K_{D,\text{diss}} \) of \(\sim 35\) \(\mu\)M, predominantly due to a marked decrease in the \(k_\text{diss} \) (\(k_\text{diss} \) values for 5.4 \(\times 10^4\) M\(^{-1}\) s\(^{-1}\) for wild type peptide and 4.2 \(\times 10^3\) M\(^{-1}\) s\(^{-1}\) for mutant peptide). Similar changes in the affinity of the interaction between peptide 11 and CaM were measured in mutant F277A/F278A. Complete abrogation of CaM binding to peptide 11 was noted when the tryptophan residue (Trp\(^{268}\)) as well as the two phenylalanine residues (Phe\(^{277}\)-Phe\(^{278}\)) in peptide 11 were replaced by alanine residues. These findings imply that the aromatic amino acids in peptide 11 of 4.1R play a critical role in Ca\(^{2+}\)-independent binding to CaM. Data obtained using r30kDa domain in which Phe\(^{277}\)-Phe\(^{278}\) was mutated to Ala\(^{277}\)-Ala\(^{278}\) or in which Trp\(^{268}\) and Phe\(^{277}\)-Phe\(^{278}\) were mutated to Ala\(^{268}\) and Ala\(^{277}\)-Ala\(^{278}\) validated the results obtained using the mutant peptide 11 (data not shown).

**Ca\(^{2+}\) and CaM Regulation of r30kDa Binding to Band 3—**Binding of 4.1R to inside-out vesicles prepared from erythrocyte membranes has previously been shown to be regulated by Ca\(^{2+}\) and CaM (18). Since the cytoplasmic domain of band 3 in inside-out vesicles has been shown to interact with the 30-kDa domain of 4.1R, we explored directly whether this interaction is regulated by Ca\(^{2+}\)-CaM. The 30-kDa domain of 4.1R bound to the cytoplasmic domain of band 3 with a \(K_{D,\text{diss}} \) value of 0.1 \(\mu\)M in the absence of CaM (data not shown). CaM alone (Table IV) or Ca\(^{2+}\) alone (data not shown) had no effect on this binding affinity. However, in the presence of both Ca\(^{2+}\) and CaM, the affinity of binding of r30kDa to band 3 decreased 10-fold (Table IV). Deletion of CaM binding motifs did not alter the intrinsic affinity of r30kDa for band 3 but did abolish the Ca\(^{2+}\)-CaM-induced decrease in binding affinity (Table IV). Wild type r30kDa, ΔEx.11, and ΔEx.9/11 all bound to cytoplasmic domain of band 3 with \(K_{D,\text{diss}} \) values ranging from 0.1 to 0.2 \(\mu\)M in the absence of Ca\(^{2+}\), but only r30kDa showed reduced binding affinity in the presence of Ca\(^{2+}\) (Table IV). Most importantly, while replacement of Ser\(^{185}\) by Trp\(^{185}\) in exon 9 of r30kDa did not alter the intrinsic affinity of the mutant protein to the cytoplasmic domain of band 3, it completely abolished the Ca\(^{2+}\)-CaM-induced decrease in binding affinity (Table IV). These results strongly imply that Ca\(^{2+}\) and CaM regulation of the interaction of 4.1R with band 3 requires both CaM binding sites and that Ser\(^{185}\) plays a critical role in modulating this interaction.

**Regulation of Band 3-Protein 4.1 Interaction by Ca\(^{2+}\) and CaM—**The Ca\(^{2+}\) concentration dependence of the CaM-modulated interaction between 4.1R and band 3 was quantitated (Fig. 4). At Ca\(^{2+}\) concentrations greater than 0.1 \(\mu\)M (\(p\text{Ca} < 7\)), the extent of r30kDa binding to band 3 started to decline, and maximal inhibition of binding was noted at Ca\(^{2+}\) concentrations of 100 \(\mu\)M and higher (\(p\text{Ca} < 4\)). Maximal effect was seen at a Ca\(^{2+}\) concentration of \(\sim 2\) \(\mu\)M.

**DISCUSSION**

The present study has identified two distinct CaM binding sites in 4.1R encoded by two different exons. These two sites exhibit interesting differences in their affinity for CaM in the presence and absence of Ca\(^{2+}\). While the site encoded by exon 11 binds CaM with high affinity in the absence of Ca\(^{2+}\), the site encoded by exon 9 binds CaM with high affinity only in the presence of Ca\(^{2+}\). The measured stoichiometry of CaM binding to 4.1R (1:1) implies that one molecule of CaM binds to two distinct binding sites in the same molecule of 4.1R. Thus, 4.1R is a unique CaM-binding protein in that it has distinct Ca\(^{2+}\)-
dependent and Ca\(^{2+}\)-independent high affinity CaM binding sites.

While in the vast majority of CaM-binding proteins the binding domain is believed to be contained in a single contiguous sequence motif, a limited number of proteins, including phosphorylase kinase and caldesmon, have been shown to contain two distinct noncontiguous CaM-binding domains (34, 35). 4.1R belongs to this latter class of CaM-binding proteins with one significant difference. In contrast to phosphorylase kinase and caldesmon, which bind CaM with high affinity only in the presence of Ca\(^{2+}\), 4.1R binds CaM with high affinity even in the absence of Ca\(^{2+}\) (11). Although 4.1R can bind CaM in a Ca\(^{2+}\)-independent manner, modulation of 4.1R interactions with its binding partners requires both Ca\(^{2+}\) and CaM (11, 16). Our finding that 4.1R possesses both a Ca\(^{2+}\)-dependent and a Ca\(^{2+}\)-independent CaM binding site and that high affinity binding of CaM to both of these sites is critical for regulation of 4.1R binding to membrane proteins provides a mechanism to explain earlier observations regarding the effect of Ca\(^{2+}\)/CaM on membrane protein interactions with 4.1R (11, 15, 16).

By quantitating the binding of CaM to various synthetic peptides that represent the two distinct CaM binding domains, we have gained insight into the nature of the interaction between CaM and 4.1R. CaM bound to peptide 9 (AKKLKSLMDLHLHAKAKDL) with 6.36  

### Table IV

| Condition   | CaM down-regulation of r30kDa-band 3 interaction |
|-------------|--------------------------------------------------|
| Wild type   | EGTA                                             |
| +Ca\(^{2+}\) | 0.18 ± 0.01                                      |
| S185 mutant | EGTA                                             |
| +Ca\(^{2+}\) | 0.12 ± 0.03                                      |
| ∆Ex.11      | EGTA                                             |
| +Ca\(^{2+}\) | 0.12 ± 0.01                                      |
| ∆Ex.9/11    | EGTA                                             |
| +Ca\(^{2+}\) | 0.28 ± 0.05                                      |
|            | EGTA                                             |
| +Ca\(^{2+}\) | 0.14 ± 0.01                                      |

| r30kDa      | Condition | K(D)kin |
|-------------|-----------|---------|
| Wild type   | EGTA      | 0.18 ± 0.01 |
| +Ca\(^{2+}\) | 2.00 ± 0.21 |
| S185 mutant | EGTA      | 0.12 ± 0.03 |
| +Ca\(^{2+}\) | 0.12 ± 0.02 |
| ∆Ex.11      | EGTA      | 0.12 ± 0.01 |
| +Ca\(^{2+}\) | 0.28 ± 0.05 |
| ∆Ex.9/11    | EGTA      | 0.14 ± 0.01 |
| +Ca\(^{2+}\) | 0.11 ± 0.05 |

\(K(D)\) represents mean ± S.D. (n = 3–5).
Fig. 6. Sequence conservation of CaM binding sites among 4.1 superfamily members. The top row shows the sequence of the Ca$^{2+}$-dependent site (peptide 9) and Ca$^{2+}$-independent site (peptide 11) in 4.1R. These CaM binding sites are highly conserved in the close homologues, 4.1G and 4.1N. Although the overall 30-kDa membrane-binding domain is highly homologous between 4.1R and the ERM proteins (ezrin, radixin, and moesin), the CaM binding sites are poorly conserved. Shaded regions indicate identical amino acids.

explain the apparent discrepancy between the present findings and the previous findings of Tanaka et al. (11), who observed only Ca$^{2+}$-independent binding of purified 4.1R to CaM and did not identify the Ca$^{2+}$-dependent binding site. The high affinity of CaM for the Ca$^{2+}$-independent binding site could have masked the Ca$^{2+}$-dependent site during measurements with native 4.1R.

Serine$^{185}$ in 4.1R was identified as the critical residue responsible for Ca$^{2+}$-sensitive binding of peptide 9 with CaM based on studies with synthetic peptides and with the entire 30-kDa domain. This conclusion is reinforced by the finding that while Ca$^{2+}$ had no effect on CaM binding to peptide 11, which does not contain the serine residue, replacement of Trp$^{268}$-Lys$^{269}$ with Ser$^{268}$-Leu$^{269}$ in this peptide induced Ca$^{2+}$-sensitive binding of peptide 11 to CaM. Our findings further suggest that nonpolar amino acids next to the serine residue, such as Met$^{186}$ in peptide 9 and Leu$^{269}$ in the mutant peptide 11, may be required for Ca$^{2+}$-sensitive binding to CaM. Aromatic amino acids, Trp$^{268}$ and Phe$^{277}$-Phe$^{278}$, appear to play a critical role in the high affinity binding of peptide 11 to CaM in the absence of Ca$^{2+}$. Further structural studies are needed to define the role of these different residues in the interaction of 4.1R with CaM.

4.1R binds to transmembrane proteins band 3 and glycoporphin C in erythrocytes. The binding site in 4.1R responsible for its interaction with band 3 is the sequence motif LEEDY (36), encoded by exon 5 (18). 4.1R binding to band 3 results in the loss of the high affinity binding sites for ankyrin on band 3 with resultant alterations in membrane mechanical properties (37). Since Ca$^{2+}$/CaM can dissociate 4.1R from band 3, they can indirectly modulate the ankyrin-band 3 interaction and hence the membrane mechanical properties (37). The effect of Ca$^{2+}$/CaM on 4.1R binding to different transmembrane proteins is yet to be quantitatively evaluated. In the present study, we document that Ca$^{2+}$ and CaM regulate the interaction of 4.1R with band 3. In the absence of either CaM or Ca$^{2+}$, r30kDa bound to band 3 with high affinity ($K_{D_{dis}}$ of 0.18 μM), but in the presence of both Ca$^{2+}$ and CaM, the affinity of interaction was reduced by an order of magnitude ($K_{D_{dis}}$ of 2.0 μM). However, Ca$^{2+}$/CaM had no effect on the affinity of the interaction between band 3 and the 30-kDa domain of 4.1R, from which either the Ca$^{2+}$-independent or both the Ca$^{2+}$-dependent and -independent CaM binding sites were deleted. These data strongly imply an absolute requirement for high affinity binding of CaM to both the Ca$^{2+}$-independent and the Ca$^{2+}$-independent sites in 4.1R for regulation of 4.1R binding to band 3 by Ca$^{2+}$ and CaM. Furthermore, the importance of Ser$^{185}$ in regulating this interaction was reinforced by the finding that replacement of Ser$^{185}$ by Trp$^{185}$ in the 30-kDa domain abolished Ca$^{2+}$/CaM-dependent regulation of 4.1R interaction with cytoplasmic domain of band 3.

Based on these findings, we propose the following model for the Ca$^{2+}$/CaM regulation of protein 4.1R interaction with band 3 in the erythrocyte membrane (Fig. 5). Ca$^{2+}$ concentration inside red cells is normally maintained at less than 1.0 μM. At this Ca$^{2+}$ concentration, CaM should bind with high affinity to the Ca$^{2+}$-independent site in peptide 11 and only weakly to the Ca$^{2+}$-dependent site in peptide 9. With increased Ca$^{2+}$ concentration, CaM binds to the Ca$^{2+}$-dependent site with high affinity, causing a conformational change in 4.1R that affects the binding sites for band 3 encoded by exon 5 (18, 36), resulting in decreased affinity of 4.1R for this transmembrane protein. Indirect evidence in support of such a CaM-induced conformational change in 4.1R has been provided by Tanaka et al. (11). However, direct evidence to support this model awaits elucidation of the structure of the membrane binding domain of 4.1R.

Recently, two homologues of 4.1R, designated 4.1G and 4.1N, have been identified and characterized (38, 39). These proteins share high degrees of sequence similarity in different functional domains, such as the 30-kDa membrane binding domain, but exhibit different patterns of tissue expression. The two CaM binding domain sequences we have identified in 4.1R are highly conserved in the homologues of 4.1R (Fig. 6), suggesting that the interactions of these newly identified proteins with their membrane partners are likely to be regulated by Ca$^{2+}$/CaM. In marked contrast, these CaM binding sequences are not conserved in other previously identified 4.1R related proteins such as ezrin (40, 41), moesin (42), and radixin (43) (Fig. 6). These findings suggest that Ca$^{2+}$/CaM regulation of protein-protein interactions is a distinct feature of 4.1R and its closely related homologues, and not a general feature of all members of the 4.1 superfamily.

4.1R and its homologues are present in a variety of nonerythroid cells and interact with a diverse group of transmembrane proteins in these cells. It is likely that these protein-protein interactions may be regulated by Ca$^{2+}$/CaM. For example, CD44, a transmembrane cell adhesion molecule, has recently been shown to bind to 4.1R in keratinocytes, and the interaction is modulated by Ca$^{2+}$/CaM (33). After ligand binding, the cytosolic Ca$^{2+}$ concentration increases, activating many Ca$^{2+}$-dependent cellular functions including those modulated by CaM. We suggest that Ca$^{2+}$/CaM regulation of the interactions between transmembrane proteins and 4.1R and its new homologues may play a major role in cytoskeletal reorganization during cell signaling in erythrocyte and nonerythroid cells.

Acknowledgments—We are grateful to M. Aoyagi of Nissei Sangyou Co., Ltd., for technical advice regarding the IAsysTM systems.

REFERENCES

1. Crivici, A., and Ikura, M. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 85–116
2. Vogel, H. J. (1994) Biochem. Cell Biol. 72, 357–376
3. Sobue, K., Muramoto, Y., Fujita, M., and Kakuchi, S. (1981) Biochem. Biophys. Res. Commun. 100, 1063–1070
4. Husain, A., Howlett, G. J., and Sawyer, W. H. (1985) Biochem. Int. 10, 1–12
5. Cohen, C. M., and Gascard, P. (1992) Semin. Hematol. 29, 244–292
6. Kessler, F., Falchetto, R., Heinz, R., Meili, R., Varherr, T., Strehler, E. E., and Carafoli, E. (1992) Biochemistry 31, 11785–11792
7. Filoteo, A. G., Eyendi, A., and Penniston, J. T. (1992) J. Biol. Chem. 267, 11800–11805
8. Varherr, T., Quadrioni, M., Krebs, J., and Carafoli, E. (1992) Biochemistry 31, 8245–8251
9. Mische, S. M., Moosker, M. S., Morrow, J. S. (1987) J. Cell Biol. 105, 2837–2845
10. Scaramuzzino, D. A., and Morrow, J. S. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 3398–3402
11. Tanaka, T., Kadowaki, K., Lazarides, E., and Sobue, K. (1991) J. Biol. Chem. 266, 1134–1140
12. Kuhlman, P. A., Hughes, C. A., Bennett, V., and Fowler, V. M. (1996) J. Biol. Chem. 271, 7966–7991
13. Larsen, F. L., and Vincenzi, F. F. (1979) Science 204, 306–309
14. Anderson, J. P., and Morrow, J. S. (1987) J. Biol. Chem. 262, 6365–6372
15. Takakuwa, Y., and Mohandas, N. (1988) J. Clin. Invest. 82, 394–400
16. Lombardo, C. R., and Low, P. S. (1984) Biochim. Biophys. Acta 1196, 139–144
17. Conboy, J. G. (1993) Semin. Hematol. 30, 58–73
18. Conboy, J. G., Chan, J. Y., Chasis, J. A., Kan, Y. W, and Mohandas, N. (1991) J. Biol. Chem. 266, 8273–8280
19. Guan, K. L., and Dixon, J. E. (1991) Anal. Biochem. 192, 262–267
20. Pasternack, G. R., Anderson, R. A., Lets, T. L., and Marchesi, V. T. (1985) J. Biol. Chem. 260, 3676–3683
21. Gopalakrishna, R., and Anderson, W. B. (1982) Biochem. Biophys. Res. Commun. 104, 830–836
22. Algrain, M., Turunen, O., Vaheri, A., Louvard, D., and Arpin, M. (1993) J. Cell Biol. 120, 129–139
23. Lankes, W. T., and Furthmayr, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8297–8301
24. An, X. L., Takakuwa, Y., Nunomura, W., Manno, S., and Mohandas, N. (1996) J. Biol. Chem. 271, 33187–33191
25. Gould, K. L., Bretscher, A., Esch, F. S., and Hunter, T. (1989) EMBO J. 8, 4133–4142
26. Algrain, M., Turunen, O., Vaheri, A., Louvard, D., and Arpin, M. (1993) J. Cell Biol. 120, 129–139
27. Parra, M., Gascard, P., Walensky, L. D., Snyder, S. H., Mohandas, N., and Conboy, J. G. (1998) Genomics 31, 145–150
28. Funayama, N., Nagafuchi, A., Sato, N., Tsukita, S., and Tsukita, S. (1991) J. Cell Biol. 115, 1039–1048
29. Zhou, N., Yuan, T., Mak, A. S., and Vogel, H. J. (1997) Biochemistry 36, 2817–2825
30. Cush, R., Cronin, J. M., Stewart, W. J., Maule, C. H., Molloy, J., and Goddard, N. J. (1993) Biosensors Bioelectronics 8, 347–353
31. Pasternack, G. R., Anderson, R. A., Lets, T. L., and Marchesi, V. T. (1985) J. Biol. Chem. 260, 3676–3683
32. Schischmanoff, P. O., Winardi, R., Discher, D. E., Parra, M. K., Bicknese, S. E., Witkowska, H. E., Conboy, J. G., and Mohandas, N. (1995) J. Biol. Chem. 270, 21243–21250
33. Tanaka, K., Waki, H., Ido, Y., and Akita, S. (1988) Rapid Commun. Mass Spectrom. 3, 151–153
34. Funayama, N., Nagafuchi, A., Sato, N., Tsukita, S., and Tsukita, S. (1991) J. Cell Biol. 115, 1039–1048
Ca^{2+}\text{-dependent and Ca}^{2+}\text{-independent Calmodulin Binding Sites in Erythrocyte Protein 4.1: IMPLICATIONS FOR REGULATION OF PROTEIN 4.1 INTERACTIONS WITH TRANSMEMBRANE PROTEINS}
Wataru Nunomura, Yuichi Takakuwa, Marilyn Parra, John G. Conboy and Narla Mohandas

J. Biol. Chem. 2000, 275:6360-6367.
doi: 10.1074/jbc.275.9.6360

Access the most updated version of this article at http://www.jbc.org/content/275/9/6360

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 43 references, 21 of which can be accessed free at http://www.jbc.org/content/275/9/6360.full.html#ref-list-1