The Interaction between Ku Antigen and REF1 Protein Mediates Negative Gene Regulation by Extracellular Calcium* (Received for publication, September 26, 1995, and in revised form, December 28, 1995)

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Through the specific binding of a negative calcium-responsive element to its binding protein in response to extracellular Ca (Ca2+)−, negative calcium-responsive element-bearing genes, such as the human parathyroid hormone gene, are negatively regulated by Ca2−. The Ku antigen mediated negative gene regulation by Ca2− by interacting with a redox factor protein, REF1. Although sequence-nonspecific DNA binding activity of the Ku antigen has been well characterized, the mechanism of its sequence-specific DNA binding remained obscure. Here, we report that the specific binding of the Ku antigen to another protein, REF1, leads to DNA-protein complex formation with a novel sequence specificity and thereby regulates gene expression.

The Ku antigen (KuAg), 1 which consists of two subunits, p70 and p80 (p86), plays a crucial role in double-stranded break repair of DNA (1–6). In this process, its ability to bind to DNA ends nonspecifically is postulated to be related to subsequent actions such as DNA recombination or unwinding (1–3). Furthermore, such binding has been reported to be directly coupled with DNA-dependent protein kinase activity, which is elicited by the putative catalytic unit of KuAg, p350 (7, 8). On the other hand, sequence-specific binding of KuAg has been demonstrated in some genes, such as the small nuclear RNA (9), T cell receptor (10), transferrin receptor (11), collagenase (12), ribosomal RNA (13, 14), and heat shock protein genes (15, 16). Although expression of most of these genes is stimulated by KuAg, transcription of the latter two is repressed by KuAg (17–19). The sequence of oligo A4 is

\[
\text{GGTAAACATCGGCGTCTTGG} \\
\text{AGCACTGGCGGCTTTGG}
\]

* This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science, and Culture of Japan and by a grant from the Japan Science Foundation of J. apant. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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We previously reported that two DNA elements located far upstream of the human parathyroid hormone gene mediated negative gene regulation by extracellular Ca (Ca2−). These DNA elements (negative calcium-responsive elements (nCaReS)) bound to common nuclear proteins (nCaREs) in a sequence-specific and Ca2−-dependent manner (17, 18). We further demonstrated that a redox factor protein, REF1, was one component of nCaREB by using the protein-DNA binding (Southwestern) assay (19). REF1 was first identified as a mammalian homologue of bacterial apurinic endonuclease repair enzyme (20). Subsequently, it was reported to potentiate DNA binding activity of several transcription factors such as AP1 and NFκB by modifying the redox state of these proteins (21). In addition to such activities of REF1, we first reported that it also possessed the sequence-specific transcriptional repressor function of nCaRE (19). However, REF1 alone could not explain all the characteristics of nCaREB activity, and we predicted the existence of another nuclear protein(s) that functions as nCaREB by cooperating with REF1 (19). By employing an oligonucleotide affinity column (22) and amino acid microsequencing (23), we demonstrate here that both subunits of KuAg interact with REF1 to bind to one of the nCaReEs and function as nCaREB.

MATERIALS AND METHODS

Synthetic Oligonucleotides and Plasmid Constructions—The sequence of oligonucleotides, oligo A and oligo SP1 site, have seen described (17–19). The sequence of oligo A4 is:

\[
\text{CCATTGTACAGCCAGAAAC} \\
\text{GCTAAAACATCGCAGCTTGG}
\]

Sequence 1.

The underlined four base pairs are completely different from those of oligo A (17). The method to construct thymidine kinase promoter-based plasmids has also been shown (17). For stable transfection of either subunit of the Ku antigen, a 2.3-kilobase pair BamHI fragment containing full-length p80 cDNA (25) or a 0.4-kilobase pair EcoRI fragment containing the 5′-coding sequence of p70 cDNA (2) was subcloned into the respective unique site of pc866 neo vector (a generous gift from S. Xanthoudakis, Roche Institute, Nutley, N.J.) in the antisense orientation (α-p80 and α-p70 plasmids). Because transfection of the latter p70-based plasmid did not reduce the amount of p70 significantly in the cultured cells, we also constructed another plasmid, which contained a 1.4-kilobase pair XbaI-Hind fragment of cDNA α-p86, pGEMTZ2(+) (2).
subcloned into the indicated sites of pRcCMVNeo (Invitrogen). The construction of pRcCMV-REF1 plasmid has been reported (19).

Oligo-A Affinity Column—In brief, 10 ml (50 mg) of Hel.a cell nuclear protein (Hnp) was mixed with 2 mg of sheared herring DNA for 15 min at 4 °C. Latex particles (25 mg) upon which 25 μg of hexamer of oligo A was immobilized were then added to Hnp for 60 min at 4 °C (22). The binding reaction was terminated by a brief centrifugation, and the particles were washed extensively five times with buffer A (20 mM Hepes (pH 7.9), 5 mM MgCl2, 20% glycerol, 0.25 mM EDTA, 0.1% Nonidet P-40 and 1 μM dithiothreitol) containing 100 mM NaCl. Subsequently, nCaREB was eluted with buffer A containing stepwise gradient concentrations (0.05–1.0 M) of NaCl, and nCaREB activity was assessed with an electrophoretic mobility shift assay (EMSA) using 10% of the eluted protein and the 32P-end-labeled oligo A as a probe. 10% of the protein eluted in 0.5 M NaCl after three consecutive passes over the column were loaded separately onto an 8% analytical SDS-polyacrylamide gel, and the proteins were visualized by silver staining. The fraction in which the two subunits of KuAg were most enriched, the eluate in 0.5 M NaCl after the third pass, was loaded onto an 8% preparative SDS-polyacrylamide gel, and amino acid microsequencing was performed as described below.

Transfection and CAT Assay—Hel.a cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. For changing Ca2+ concentrations of the medium, either EGTA (to the same medium) or calcium chloride (to the calcium-free Dulbecco's modified Eagle's medium/Ham's F-12 containing 10% fetal calf serum) was employed. In the CAT assay, 5 μg of the reporter plasmid was transiently cotransfected with 2.5 μg of both the above antisense-oriented KuAg expression vectors or with 5 μg of the vector alone. The method for CAT assay has been described (17–19).

Amino Acid Microsequencing—In the preparative SDS-polyacrylamide gel, 50 ng of Hnp yielded 400 ng of each of the purified proteins. The purified proteins were transferred onto a polyvinylidene difluoride membrane, stained with Ponceau S, and excised. Then, the automated amino acid sequencing was carried out. To obtain the internal sequences of the proteins, the similarly treated and transferred proteins were subjected to leucine peptidase digestion, and then the digested proteins were eluted and separated by HPLC before automated amino acid microsequencing (23). The computer search for the protein containing the sequence was performed using the Swissprot program.

Preparation of Nuclear Extracts and EMSA—The method for obtaining nuclear extracts from wild-type Hel.a cells or Hel.a cells transfected with the expression vectors and for EMSA have been described (17–19). When purified KuAg (7, 26) was used, 40 ng of it was included in the reaction. Incubation time was 30 min unless otherwise indicated. In lane 1, 2, 6, 7 and 9 in Fig. 3A, 10 μg of the nuclear protein from the indicated cells were used, and in lanes 2, 5, and 8 in the same figure, 5 μg of the proteins from each of the surrounding lanes were mixed, and “complementation EMSA” was performed. When the polyclonal anti-REF1 and anti-KuAg antibodies were used, 1 μl of each of them was added in the reaction for the final 10 min. In Fig. 4B, after preincubation of 10 μg of Hnp and 1 μl of the Ab at room temperature for 10 min, 200 ng of both b-REF1 (or its truncated form) and labeled oligo A were included in the mixture for the final 20 min, and EMSA was carried out. In this experiment, a 6% nondenaturing polyacrylamide gel was used to examine whether a complex(es) migrating at the position lower than complex A would appear. All of the other EMSA in this paper were performed by using 4% gels.

Construction of Truncated REF1 and Protein-Protein Interaction—REF1 genes truncated at the amino terminus were constructed by polymerase chain reactions using oligonucleotides corresponding to fixed 3' and variable 5' ends points. Carbonyl-terminal truncations of REF1 were generated by polymerase chain reaction using oligonucleotides corresponding to fixed 5' and variable 3' ends points (N120, positions 121–318; C107, positions 1–211). All the polymerase chain reaction products were digested with BamHI and HindIII. Digestted fragments were inserted into pGEX4-3 (27). The truncated protein genes of these constructs were expressed from the GGA triplet of the GGTACC BamHI recognition sequence as fusion proteins. All resultant plasmids were introduced into Escherichia coli (M109). The truncation series (b-REF1) was expressed as glutathione S-transferase (GST) fusion proteins after isopropyl-1-thio-β-D-galactopyranoside induction. For the interaction between the purified KuAg (7, 26) and the above b-REF1s, 20 ng of the former and 200 ng of the latter were mixed for 5 min on ice, washed with phosphate-buffered saline in the presence of GST-agarose three times, and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblotting with the anti-KuAg antibody (7, 26) was performed by the enhanced chemiluminescence method, as reported (19).

RESULTS

Purification of nCaREB—Although nCaRE bound to purified REF1, nCaREB contained at least one another nuclear protein (19). However, we had failed to clone any nCaRE other than REF1 by the Southwestern method. We assumed that this failure might be due to the inability of the protein(s) to bind to nCaRE in the denaturing/renaturing process. Thus, we tried to purify nCaREB in Hnp by employing a column purification method that used latex particles (22) onto which tandemly immobilized oligo A was added in the reaction for the final 10 min. In Fig. 1A, the elution profile of nCaREB was carried out as described above. Incubation time was 30 min unless otherwise indicated. In Fig. 4B, after preincubation of 10 μg of Hnp and 1 μl of the Ab at room temperature for 10 min, 200 ng of both b-REF1 (or its truncated form) and labeled oligo A were included in the mixture for the final 20 min, and EMSA was carried out. In this experiment, a 6% nondenaturing polyacrylamide gel was used to examine whether a complex(es) migrating at the position lower than complex A would appear. All of the other EMSA in this paper were performed by using 4% gels.

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hand, the two proteins of 70 and 80 kDa were consistently co-purified through many trials. In the subsequent preparative SDS-polyacrylamide gel, we found that 50 mg of Hnp yielded 400 ng of each of the proteins. Table I shows typical results of the purification. Amino acid microsequencing (Ref. 23; see “Materials and Methods”) revealed three short peptides from each band (Fig. 1C), all of which coincided with partial amino acid sequences of the two subunits of KuAg, p70 and p80 (24, 25).

Interaction between KuAg and Oligo A—Both subunits of KuAg were prepared and purified from serum of a patient of scleroderma/polymyositis by phosphocellulose and double-stranded DNA-Sepharose chromatography and anion exchange HPLC as reported (7, 26). As shown in Fig. 2A, they could form one discrete complex (complex A) with oligo A in EMSA. Complex A was considered identical to the fastest migrating band (complex A) among several complexes generated by the interaction between Hnp and oligo A (Fig. 2A). First, its migrating position on the gel was identical to that of complex A. The addition of the purified KuAg to Hnp did not produce another bands even after longer electrophoresis (not shown). Second, the anti-KuAg antibody (7) abolished complex A formation, whereas preimmune serum did not. Third, one of the mutants of oligo A, oligo A4, in which the central four bases of oligo A were completely altered, did not inhibit complex A formation. Such sequence specificity observed in complex A formation paralleled that seen in complex A (Fig. 2A). However, we noticed that even a 50-fold molar excess of oligo A4 did not abolish the formation of complex A, although a molar excess greater than 10-fold eliminated complex A. Thus, sequence-specific binding of the purified KuAg, unlike that of Hnp, could be seen only in a narrow range of a quantitative ratio between KuAg and the probe DNA (9–16, see “Discussion”).

However, besides complex A, Hnp produced several slower migrating complexes bound to oligo A. Shorter incubations generated an uppermost band (complex B) that behaved similarly to complex A in terms of sequence specificity (Fig. 2B). Time course EMSA experiments revealed that 5 min of incubation generated almost equal intensities of both complexes A and B (Fig. 2B). Longer incubation led to the formation of stronger complex A and fainter complex B, resulting in almost complete disappearance of complex B after 20 min of incubation. This observation suggested that complex B was first generated and then converted to complex A.

Effect of Antisense-oriented Expression of the p80 Subunit of KuAg—We next modified HeLa cells by stable transfection with a p80 subunit expression vector in the antisense orientation (α-p80 cells) in order to lower the amount of p80 in the cells. Immunoblot analysis revealed that the amounts of both p80 and p70 were decreased in these cells (Fig. 2C; see “Discussion”). By densitometoric scanning, we found that only 20% of p80 and 50% of p70 were retained in these cells compared with those in control cells. Interestingly, as shown in EMSA in Fig. 2D, like complex A, complex B was markedly diminished in these cells, whereas complexes between such Hnp and SP1 site (C1 and C2) were not affected.

Interaction between KuAg and REF1 Protein: Complementation EMSA—Because REF1 protein had nCaREB activity (19), generation of these complexes might be affected after reducing the amount of REF1 protein by a similar antisense strategy. As
Fig. 3. Complementation EMSA and effects of Ca2+ on the complexes in EMSA. A, effects of mixing of Hnp in EMSA. Hnp from Fer cells (19) was mixed either with that from α-p80 (lanes 1-6) or with that from α-p70 cells (lanes 7-9). In the lanes denoted as M (lanes 2, 5, and 8), half the amount of Hnp used in the respective surrounding lanes was mixed and subjected to EMSA. In lanes 1-3 and 7-9 oligo A and in lanes 4-6 SP1 site were used as radiolabeled probes. B, effects of Ca2+ on the complexes in EMSA. Oligo A was used as a probe. In both A and B, EMSA was carried out after 10 min of incubation of the reaction mixture.

shown in Fig. 3A, Hnp from antisense REF1-transfected cells (lane 1, Fer cells; Ref. 19) as well as that from α-p80 cells (lane 3) produced overall weak EMSA bands. Importantly, mixture of half the amount of the protein used in each lane (lane 2) conferred more than additive effects on the two bands. Although the bands between the complexes A and B behaved similarly to complex A in some experiments, they did not reproducibly appear. On the other hand, such mixture gave just additive effects on the binding between the SP1 site and its binding proteins (Fig. 3A, lanes 4–6).

Then we attempted to establish α-p70 cells by stable transfection of a p70 subunit expression vector in the antisense orientation in order to generate Hnp that would contain reduced amounts of the p70 subunit. However, all the clones examined yielded only marginally reduced amounts of the p70 protein (Fig. 2C; see “Discussion”). Nonetheless, we thought these differences were significant, because the intensities of the bands other than p70 or p80 were comparable (Fig. 2C, lanes 1 and 2). Further, in EMSA, the intensity of complex B was never attenuated, and that of complex A was slightly attenuated in Hnp from these α-p70 cells (Fig. 2D). Perhaps related to this phenomenon, we observed that a similar mixture of Hnp from any one of these α-p70 cells with that from Fer cells conferred just additive but not synergistic effects on complex B formation, whereas effects on complex A were again synergistic (Fig. 3A, lanes 7–9). These results suggest that even a small decrease in the amount of p70 in Hnp from our α-p70 cells might be sufficient for evaluating potential functional cooperativity of p70 with REF1. Taken together, we assume that REF1 and KuAg interact in a highly cooperative manner but that the p70 subunit might not participate in the p80-REF1 complex (complex B) especially in the early time point.

Effect of Ca2+ on the Oligo A-nCaREB Binding—We next examined whether complex B contained KuAg. However, we failed to erase or supershift complex B formation by the anti-KuAg Ab in EMSA (Fig. 2A and data not shown); this result is in sharp contrast to that following the use of the anti-REF1 Ab (Fig. 2B). It might reflect a possible conformational change due to the interaction between p80 and REF1, which would prevent an access of the anti-KuAg Ab to the complex. Nonetheless, both complexes A and B were similarly up-regulated by a rise in Ca2+ concentration (Refs. 17–19; Fig. 3B).

Protein-Protein Interaction between KuAg and REF1: Binding Domain within REF1 Protein—We next made a truncated series of bacterially produced REF1 (Ref. 19; b-REF1s) tagged with GST (27). As shown in the upper panel of Fig. 4A, full-length p70 complex A on the reappearance of complex A after suppression by the addition of the anti-REF1 Ab (19). As a control, complex A+ was included. Oligo A was used as a probe. After preincubation of 10 μg of Hnp and 1 μl of the Ab at room temperature for 10 min, 200 ng of each b-REF1 and labeled oligo A were added to the mixture for the final 20 min, and EMSA was carried out. A 6% nondenaturing polyacrylamide gel was employed to examine whether a band(s) other than complex A was formed.

Fig. 4. Interactions between KuAg and REF1. A, interactions between the purified KuAg and a truncated series of b-REF1 tagged with GST. 20 ng of KuAg and 200 ng of each b-REF1 or GST alone were briefly incubated on ice, and then the mixture was trapped by the GST-agarose followed by a 10% SDS-polyacrylamide gel electrophoresis. Equal amounts of the trapped proteins were subjected either to immunoblotting using the anti-KuAg Ab (upper panel) or to silver staining (lower panel). In the lower panel, the positions of each of the truncated or full-length b-REF1s were indicated by triangles. B, effects of truncated b-REF1s on the reappearance of complex A after suppression by the addition of the anti-REF1 Ab (19). As a control, complex A+ was included. Oligo A was used as a probe. After preincubation of 10 μg of Hnp and 1 μl of the Ab at room temperature for 10 min, 200 ng of each b-REF1 and labeled oligo A were added to the mixture for the final 20 min, and EMSA was carried out. A 6% nondenaturing polyacrylamide gel was employed to examine whether a band(s) other than complex A was formed.

The results in Fig. 4(A and B) suggest that the NH2-terminal portion of REF1 is crucial for its synergistic interaction with KuAg, although we could not specify which subunit of KuAg was involved here, because separation of KuAg into each subunit was difficult in these experiments.
REF1 augments DNA binding activity of several transcriptional factors through some of its cysteine residues, especially Cys65, by modifying redox states of these proteins (21, 30). Interestingly, Cys65 is contained within the region capable of interacting with KuAg (Fig. 4, A and B).

Components of nCaREB Required for the Binding to Oligo A—Although we demonstrated that REF1 was required for formation of complex A, the migrating position of complex A in EMSA was identical to that of complex A', which contains only the two subunits of KuAg (Fig. 2A). However, not only the anti-KuAg antibody but also anti-REF1 antibody impaired complex A formation (Ref. 19; Fig. 2B). Further, the addition of full-length REF1 as well as some truncated b-REF1 led to the reappearance of the once suppressed complex A, but the position of the resultant complex A on the gel was the same regardless of the heterogeneous size of b-REF1s (Fig. 4B). Therefore, we hypothesize that there are two distinct steps in complex formation. At first, REF1 and p80, but not p70, take part in the formation of complex B, although there must be other unidentified components or multimer forms in complex B, because it migrates slowly. Then p70 participates in the complex. During EMSA, REF1 might be detached from the complex, resulting in the formation of complex A. Such a dissociation of a protein(s) from certain protein-DNA complexes in EMSA has been reported in several cases (Refs. 28 and 29; see "Discussion").

Effect of KuAg on nCaRE-mediated Transcription—We next transiently transfected HeLa cells with the reporter plasmid, oligo A-thymidine kinase CAT (17, 18) and both of the antisense-oriented p70 and p80 expression vectors (Fig. 5). Ca2\(^{2+}\) \(-\) dependent suppression of the CAT activity driven by oligo A-thymidine kinase CAT was abrogated after reducing KuAg expression by the antisense strategy, whereas the CAT activity driven by parental thymidine kinase CAT was unaffected either by high Ca2\(^{2+}\) concentration (17–19) or by the antisense-oriented expression of KuAg. Transfection of either p70 or p80 expression vector alone in the antisense orientation did not significantly affect Ca2\(^{2+}\) \(-\) mediated suppression of CAT activity by the oligo A-bearing plasmid (data not shown). These results also underscore the functional contribution of KuAg to nCaRE-mediated gene repression by Ca2\(^{2+}\) \(-\).

**DISCUSSION**

**Ku Ag, Alone or in Association with REF1, Binds to Oligo A in a Sequence-specific Manner**—The manner of the binding of KuAg to oligo A described in this manuscript is clearly distinct from that of its well characterized nonspecific binding to DNA ends (1–5). First, unlike the predictions of previous models of the nonspecific DNA binding of KuAg, such as a sliding model (4), longer incubation time generated faster migrating complexes but did not yield slower migrating nor multiple complexes in EMSA (Fig. 2B). Second, Hnp protected oligo A, which lies in the central, but not in the terminal portion of the 110-base pair fragment, from DNase digestion (17). Binding to the ends of DNA molecules has been thought to be a hallmark of sequence-nonspecific binding of KuAg.

We also presented several lines of evidence that Hnp that specifically bound to oligo A contained not only both subunits of KuAg but also REF1 protein (Figs. 2B, 3A, 4A, and 4B). Thus, as discussed below, KuAg might well bind to oligo A in a cooperative manner with REF1, thereby giving rise to a reforced binding specificity, although the binding of the purified KuAg alone to oligo A showed less stringent but distinct sequence specificity (complex A', Fig. 2A). The sequence of oligo A does not have significant homology with other reported KuAg-responsive element, except for the octamer-like sequence within the small nuclear U1 RNA gene (7); oligo A can be considered a degenerate inverted repeat of the octamer sequence (19).

**nCaREB Consists of KuAg and REF1**—We demonstrated that both of the two nCaRE-nCaREB complexes (A and B) contained KuAg and REF1. In our transfection experiments, all the clones containing the p70 subunit in the antisense orientation exhibited a marginal reduction of the amount of the p70 protein (Fig. 2C). This is in sharp contrast to the situation in A-p80 cells (Fig. 2C), in which the amount of p80 was markedly reduced. This finding might reflect a more crucial function of p70 for the cultured cells compared with that of p80. In any case, we found that such a minimal but selective reduction of p70 led to reduced intensity of complex A but not complex B in EMSA (Fig. 2D). Along with this observation, our complementation EMSA experiments revealed a functional cooperativity of p70 with REF1 for the formation of complex A but not complex B (Fig. 3A).

On the other hand, we suggested that by the similar complementation EMSA (Fig. 3A), p80 could, separately from the p70 subunit, bind to and function with REF1 during complex B formation. Such a dissociation seems to contradict many reports (1–16) describing that many functions of KuAg were elicited by the association of p70 with p80 (and/or p350, a catalytic unit of KuAg). Nevertheless, in the time course EMSA experiment (Fig. 2B), we demonstrated a shift of complex formation from B to A, which contained both of the subunits. Furthermore, an abrogation of oligo A-mediated transcriptional repression by Ca2\(^{2+}\) \(-\) was observed only in the case of combined transfection of the p70 and p80 expression vectors in the antisense orientations (Fig. 5). Thus, the reassociation of both subunits of KuAg is predicted to lead to transcriptional suppression by Ca2\(^{2+}\) \(-\).
to oligo A (Fig. 2, A and B). The supershift (Fig. 2B) and complementation (Fig. 3A) in EMSA convincingly suggested that REF1 affected the formation of both complexes. Although we could clearly show that the purified KuAg and REF1 interacted with each other (Fig. 4A), mixture of both of them in EMSA did not create such a band as complex B migrating more slowly than complex A' (data not shown).

REF1 may be easily detached from the REF1-KuAg complex during electrophoresis in nondenaturing conditions. This hypothesis could explain why complex A migrated at the identical position with complex A' in EMSA (Fig. 2A). Further, removal of REF1 by electrophoresis could explain how the addition of some of the b-REF1s reversed the suppression of complex A without changing the mobility of complex A, irrespective of the heterologous size of b-REF1s (Fig. 4B).

The relatively short half-life of complex B (Fig. 2B) might also reflect labile binding of REF1 protein during electrophoresis. Perhaps another unidentified protein(s), such as TATA box binding protein, which has been reported to interact with KuAg to regulate the expression of the collagen gene (12), can stabilize complex B transiently. Such a dissociation during electrophoresis in EMSA has been described for NF-κB (28) and Phox (29) proteins.

Domain Structures of REF1-KuAg Binding—We found that the amino-terminal portion of REF1 bound to KuAg to function as nCaREB (Fig. 4, A and B). Further, at the putative leucine zipper motif (residues 395–399) of the p70 subunit of KuAg, there is a sequence, AALCR (residues 395–399), that is similar to the consensus site AA(K/E/R)CR needed for REF1 interaction (21). This consensus sequence found in JUN protein has been shown to be important for its binding to REF1 (30). Also, a cysteine preceded by lysine was found in the putative leucine zipper region of p80 (residues 156 and 157).

Although functional separation of KuAg into separate sub-units is difficult (1–6), our observation raises the possibility that some form of REF1-KuAg interaction really occurs in vivo. This finding is of particular interest because both REF1 and KuAg have been reported to be engaged in the basic transcriptional activity coupled with DNA repair/recombination processes (1–6, 20).

We had previously shown that not only oligo A but also oligo B functioned as nCaREs. Oligo B forms a unique palindrome sequence, and its sequence is widely conserved among several genes to achieve negative calcium responsiveness (18). Although oligo B, like oligo A, bound to REF1 protein (17–19), oligo B-specific binding to REF1-KuAg complexes could not be demonstrated (data not shown). The mechanism underlying this distinction is currently unknown.

A rise in Ca\(^{2+}\) concentration augmented the binding of nCaRE to nCaRE (Refs. 10 and 19; Fig. 3B). In response to a rise in Ca\(^{2+}\) concentration, the amounts of both mRNA and protein of REF1 were elevated (19), whereas those of KuAg were not significantly altered (not shown). We are currently examining the possibility that post-transcriptional modifications of KuAg by Ca\(^{2+}\) also play some role in potentiating nCaRE-nCaRE interactions, although it is possible that the rate-limiting step is Ca\(^{2+}\)-dependent change in the activity of REF1 protein alone.

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