Interaction of a 43-kDa Receptor-like Protein with a 4-kDa Hormone-like Peptide in Soybean†

Kazuki Hanada and Hisashi Hirano*

Graduate School of Integrated Science, Kihara Institute for Biological Research, Yokohama City University, Maioka-cho 641-12, Totsuka, Yokohama 244-0813, Japan

Received March 1, 2004; Revised Manuscript Received June 22, 2004

ABSTRACT: A 43-kDa soybean protein is a receptor-like protein kinase that is capable of interaction with a 4-kDa hormone-like peptide (leginsulin). The 43-kDa protein consists of α and β subunits; the β subunit has protein kinase activity that is stimulated by the binding of the 4-kDa peptide. The protein kinase activity is believed to be an early step in a signal transduction cascade, triggered by the peptide. Animal insulin also interacts with the 43-kDa protein and stimulates the protein kinase activity, suggesting that the 4-kDa peptide and insulin bind to the 43-kDa protein with similar mechanisms. To determine the mechanism of interaction between the 4-kDa peptide and 43-kDa protein, we investigated the binding region of the 4-kDa peptide on the 43-kDa protein using surface plasmon resonance (SPR) spectroscopy. We found that the N- (amino acids 1−43) and C-terminal (amino acids 228−251) regions of the α subunit of the 43-kDa protein are involved in the binding. The interactions of both insulin and the 4-kDa peptide with the 43-kDa protein were compared using SPR spectroscopy, revealing that insulin binds to the C-terminal regions of the α subunit of the 43-kDa protein. These results suggest that the C-terminal region is especially important for the biological function. The N-terminal region is thought to play an important role in stabilizing the complex of the 43-kDa protein and the 4-kDa peptide.

In animals and yeast, growth, differentiation, and cell proliferation are controlled by various peptide factors (*1*, *2*). In general, these peptides interact with receptor proteins located on the cell surface and stimulate the protein kinase activity of the receptors (*3*). Historically, the phytofactors that affect growth, differentiation, and cell proliferation have been believed to be organic compounds of low molecular mass (*4*). In the past decade, several plant peptide factors have been shown to act as hormones, regulating growth, differentiation, and cell proliferation (*5*, *6*).

The 4-kDa peptide (leginsulin)[1] is a hormone-like peptide found in germinating radicles of soybean seeds (*7*). Callus tissue cultured in medium containing this peptide grows more rapidly than in medium lacking the peptide, suggesting that the 4-kDa peptide may regulate growth, differentiation, and cell proliferation (*8*). The 4-kDa peptide consisting of 37 residues contains a T-knot motif (*9*), like some animal growth factors (*9*). The 4-kDa peptide may therefore be similar in function to these animal growth factors.

A soybean 43-kDa protein that is localized in cell walls and plasma membranes has protein kinase activity (*10*−*12*) that is stimulated by the binding of the 4-kDa peptide (*7*). The 4-kDa peptide may therefore initiate a signal transduction cascade, mediated by the protein kinase activity of the 43-kDa protein. Komatsu et al. (*12*) demonstrated that tyrosine phosphorylation activity is associated with the 43-kDa protein in the only report that has described tyrosine phosphorylation via protein tyrosine kinase in plants. The 43-kDa protein may be a useful tool for investigating the signal transduction systems for growth, differentiation, and cell proliferation that are unique to plants.

The 43-kDa protein consists of α (27 kDa) and β (16 kDa) subunits linked by a disulfide bridge or bridges (*13*). The α subunit is thought to interact directly with the ligand, and the β subunit has the protein kinase activity that is activated by the binding of the 4-kDa peptide (*12*). We have previously reported on the residues of the 4-kDa peptide that are involved in the interaction with the 43-kDa protein (*14*). However, in that study, we did not determine the subunit or the regions of the subunit that interacts with the 4-kDa peptide. To determine the ligand-binding region in the 43-kDa protein, we expressed fragments of the 43-kDa protein in *Escherichia coli*, purified the fragments, and investigated the interaction of the 4-kDa peptide with these fragments using surface plasmon resonance (SPR)[2] spectroscopy.

† This work was supported in part by Grants-in-Aid for National Project on Protein Structure and Functional Analyses to H.H.

* To whom correspondence should be addressed: Graduate School of Integrated Science, Kihara Institute for Biological Research, Yokohama City University, Maioka-cho 641−12, Totsuka, Yokohama 244-0813, Japan. Telephone: +81-45-820-1904. Fax: +81-45-820-1901. E-mail: hirano@yokohama-cu.ac.jp.

[1] Because of the similarities between the binding of insulin and the 4-kDa peptide to that of the 43-kDa protein, Watanabe et al. named the 4-kDa peptide leginsulin (*7*). The naming of the peptide as leginsulin is controversial. To avoid confusion, in this paper, we refer to the peptide as "4-kDa peptide".

[2] Abbreviations: Con A, concanavalin A; GF, gel filtration; HBS, HEPES-buffered saline; HPLC, high-performance liquid chromatography; IEX, ion exchange chromatography; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-β-thiogalactoside; IR, insulin receptor; PVDF, poly(vinylidene difluoride); SPR, surface plasmon resonance; TBS, Tris-buffered saline.

10.1021/bi049587a CCC: $27.50
Published on Web 08/31/2004

EXPERIMENTAL PROCEDURES

Materials. All oligonucleotides were obtained from Invitrogen Life Technologies (Carlsbad, CA). The *E. coli* expression vectors, pET-15b and pET-32a[+], the expression host cell, BL21*trxB* [DE3], and the BugBuster protein extraction reagent were from Merck (Darmstadt, Germany). An immobilized metal affinity chromatography column, HiTrap chelating HP (1 mL), was from Amersham Biosciences (Uppsala, Sweden). Biacore sensor chips CM5 and SA were from Biacore (Uppsala, Sweden). The restriction enzymes *Bam*HI, *Eco*RI, *Nco*I, and *Nde*I were from Nippon Gene (Tokyo, Japan). All other inorganic and organic compounds were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Construction of His-Tag and Trx-Tag Fusion Protein Expression Vectors. All DNA fragments of the gene encoding the 43-kDa protein α subunit were amplified by PCR and fused between the *Nde*I and *Bam*HI sites of the hexahistidine tag (His-tag) fusion protein vector pET-15b or between the *Nco*I and *Eco*RI sites of the thioredoxin/hexahistidine/S-peptide multiple tag (Trx-tag) fusion protein vector pET-32a[+]. Diagrams of the amplified fragments of the 43-kDa protein are shown in Figure 1A. In-frame cloning was confirmed by dideoxy sequencing.

Purification of His-Tag and Trx-Tag Fusion Proteins. All expression vectors, constructed as described above, were introduced into the *E. coli* strain BL21*trxB* [DE3]. The His-tag fusion proteins were prepared as follows. *E. coli* cells transformed with the expression vector were grown at 37 °C in 1 L of LB medium containing 50 μg mL^{-1} of carbenicillin, to an optical density of 0.6 at 600 nm. After addition of isopropyl-β-thiogalactopyranoside (IPTG) to a final concentration of 1.0 mM, the cells were grown for an additional 16 h at 18 °C and harvested by centrifugation at 6000*g* for 15 min at 4 °C. The cells were suspended in 20 mL of BugBuster protein extraction reagent, and the cell suspension was rotated slowly on an orbital shaker for 10 min at room temperature, after which the suspension was centrifuged at 50000*g* at 4 °C for 15 min. The His-tag fusion protein in the supernatant was captured using immobilized metal affinity chromatography (IMAC) with Ni^{2+}. After capture, the protein was purified using ion exchange chromatography (IEX).

Trx-tag fusion proteins were purified by IMAC and IEX. *E. coli* cells transformed with the expression vector were grown at 37 °C in 1 L of LB medium containing 50 μg mL^{-1} of ampicillin until the culture reached an optical density of 0.6 at 600 nm. After addition of IPTG to a final concentration of 0.7 mM, the cells were grown for an additional 4 h at 37 °C and harvested by centrifugation at 6000*g* for 15 min at 4 °C; the pellet was suspended in 20 mL of BugBuster protein extraction reagent. After incubation of the cell suspension, the cell debris was removed by centrifugation at 50000*g* at 4 °C for 15 min. The supernatant was subjected to IMAC column chromatography; the fractions containing the fusion protein were combined; and the fusion protein was purified using IEX. The purity of the proteins was monitored by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). The molecular weights of purified proteins were measured on MALDI−TOF mass spectrometer (Tofspec 2E; Micromass, Manchester, U.K.).

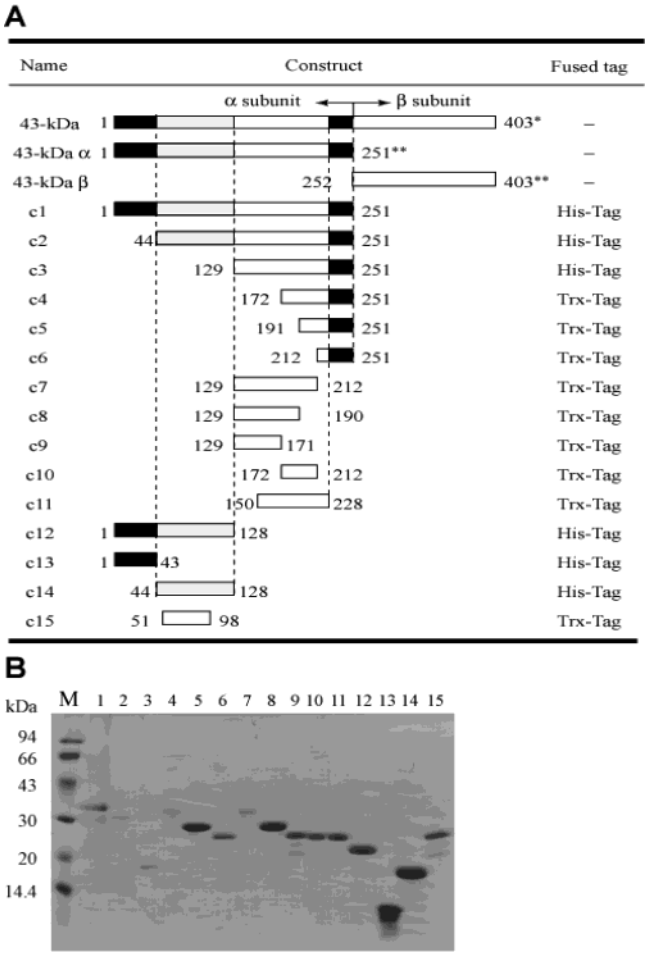

FIGURE 1: Binding of the 4-kDa peptide to fragments of the 43-kDa protein α subunit. (A) Diagram of expression constructions for fragments of the 43-kDa protein α subunit and summary of the binding results. Black areas show the most important areas for binding to the 4-kDa peptide. A single asterisk indicates that the construct was purified using the method of Yamauchi et al. (*13*). A double asterisk indicates that the construct was purified using the method of Sato et al. (*17*). (B) Coomassie-Blue-stained SDS−PAGE gel containing the expressed fragments. Lane labels (lane 1−15) correspond to the constructs, c1−15, respectively. Lane M shows a molecular-marker standard.

Production of Alanine-Substituted Mutants of Soybean 4-kDa Peptide. Alanine-substituted mutants of soybean 4-kDa peptide (I25A, F28A, V29A, F31A, and I33A) were generated using the methods of ref *14*. The fused tag was removed with recombinant enterokinase (Merck), and the 4-kDa peptide mutants were purified by reverse-phase C_{18} HPLC column (Develosil-ODS-UG5; Nomura Chemical, Seto, Japan) eluted at 0.1 mL min^{-1} with a gradient of water, 0.1% trifluoroacetic acid/acetonitrile, and 0.1% trifluoroacetic acid (20:50 in 30 min).

Ligand Blot Analysis. The 43-kDa protein was purified as described in ref *17*, subjected to SDS−PAGE under reducing conditions to separate the α and β subunits, and electrotransferred onto a poly(vinylidene difluoride) (PVDF) membrane. The membrane was incubated at room temperature, first for 5 min in Tris-buffered saline (TBS) (20 mM Tris-HCl at pH 7.4 and 0.5 M NaCl), then for 1 h in TBS containing 1% (w/v) nonfat dry milk, and finally twice for 10 min in TBS. The 4-kDa peptide was dissolved to 5 μg mL^{-1} in TBS and incubated with the membrane for 3 h at 4 °C. The membrane was washed twice with TBS for 5 min

and incubated for 1 h at 4 °C with rabbit anti-(4-kDa peptide) polyclonal antibody dissolved in TBS. The membrane was then washed twice with TBS for 5 min, incubated for 1 h at 4 °C with goat anti-(rabbit IgG) antibody dissolved in TBS, and washed twice with TBS for 5 min. Signals were detected using BCIP/NBT membrane phosphatase substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

SPR Spectroscopy. SPR spectroscopy was performed using a Biacore X instrument (Biacore). The sensor chip CM5 for the Biacore X has two flow cells (FCs). FC1 was filled with a blank solution, and 2250 RU of the 4-kDa peptide purified from soybean seeds by the methods of Watanabe et al. (*7*) or 1557 RU of insulin were immobilized in FC2. Preliminary studies showed that the 4-kDa peptide and insulin were able to be regenerated with 20 mM HCl; thus, 20 mM HCl was used as a regeneration solution. Biotinylated concanavalin A (Con A) was immobilized onto a sensor chip SA, which had streptavidin bound to its surface. NaOH (10 mM) was used as a regeneration solution for Con A. His-tagged N-terminal region (amino acids 1−43) or Trx-tagged C-terminal region (amino acids 215−251) of 43-kDa protein α subunit were coupled onto sensor chip CM5. These sensor chips were regenerated with 10 mM glycine-HCl at pH 2.0.

Three concentrations of analytes in HEPES-buffered saline (HBS) (10 mM HEPES at pH 7.4 containing 150 mM NaCl, 1 mM EDTA·2Na, and 0.005% surfactant P-20) were passed through FC1 and FC2 in series at 20 μL min^{-1} for ∼2−4 min at 25 °C. Association of analytes was monitored by SPR. Dissociation was analyzed for 3 min by passing HBS through the cells and monitoring with SPR. After each analysis, the sensor chips were regenerated with the appropriate regeneration solution and equilibrated with HBS before the next analysis. Association and dissociation rate constants (k_a and k_d) were calculated using BIAevaluation 3.2 RC2 (Biacore). The dissociation constant K_D was calculated using the formula $K_D = k_d/k_a$.

Sugar Chain Detection. The ECL glycoprotein detection system (Amersham Biosciences) was employed to determine if the 43-kDa protein was glycosylated. The α and β subunits of the purified 43-kDa protein were separated by SDS−PAGE under reducing conditions and transferred to a PVDF membrane. The 43-kDa protein was treated with biotin to label any neutral sugars. Biotin was detected using chemiluminescence with streptavidin-horseradish peroxidase.

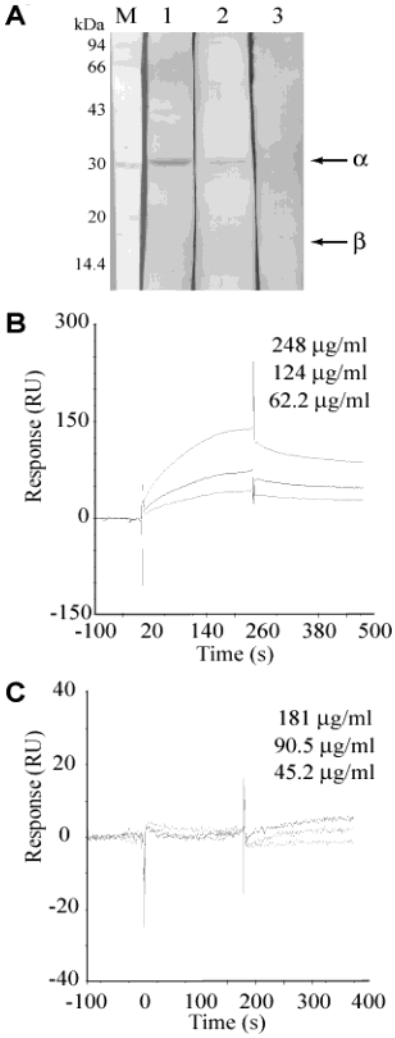

FIGURE 2: Binding of the 4-kDa peptide to subunits of the 43-kDa protein. (A) Ligand blot analysis. Lane M, molecular markers; lane 1, detection of the α subunit of the 43-kDa protein using anti-(43-kDa protein α-subunit) antibody; lane 2, detection of the 4-kDa peptide using anti-(4-kDa peptide) antibody; and lane 3, negative control using anti-(rabbit IgG) antibody. Arrows indicate the α and β subunits of the 43-kDa protein. For details, refer to the Experimental Procedures. (B and C) Representative sensorgrams showing interactions with the 4-kDa peptide. (B) 43-kDa protein α subunit. (C) 43-kDa protein β subunit. The concentrations of analytes injected into the sensor chip are indicated to the right of the sensorgrams.

RESULTS AND DISCUSSION

The 4-kDa Peptide Interacts with the α Subunit of the 43-kDa Protein. The 4-kDa peptide can bind to the 43-kDa protein, which consists of α and β subunits. We performed ligand blot analysis to determine which subunit of the 43-kDa protein interacts with the 4-kDa peptide. The α and β subunits of the 43-kDa protein were separated by SDS−PAGE under reducing conditions and transferred onto a PVDF membrane. The membrane was incubated with the 4-kDa peptide, and any interaction with the 4-kDa peptide was detected using anti-(4-kDa peptide) antibody (Figure 2A).

In a parallel approach, interaction was also examined by SPR spectroscopy. The 4-kDa peptide was immobilized in the flow cell of a sensor chip CM5. The α and β subunits of the 43-kDa protein, purified from mature soybean seeds, were applied to the chip. The binding of the 4-kDa peptide to the α subunit was found to be specific, strong, and stable (Figure 2B), with a dissociation constant (K_D) of 8.98×10^{-8} M. In contrast, the β subunit had no affinity for the 4-kDa peptide. We concluded that the α subunit of the 43-kDa protein contains the 4-kDa-peptide-binding interface.

It is known that the 4-kDa peptide can stimulate the protein kinase activity of the β subunit of the 43-kDa protein (*7*). Therefore, it is possible that the 4-kDa peptide first binds to the α subunit of the 43-kDa protein and then, through a conformational change, the kinase activity of the β subunit is activated.

Production of Fragments of the 43-kDa Protein Fused with a His or Trx Tag. As shown in Figure 1A, a total of 15 fragments were generated as fusion proteins to His or Trx tags, derived from the pET-15b or pET-32a[+] vectors, respectively. Fragments c4−11 were expressed as Trx-tag

N-terminal region

```
                    .    . :::** :* **  : : :::****:   ::.**.
soybean         VTPTKP-INLVVLPVQNDGSTGLHWANLQKRTPLMQVPVLVDLN
lupin           SQPTSSKPNLLVLPVQEDASTGLHWANIHKRTPLMQVPLLLDLN
carrot          QAQPSFRPSALVVPVKKDASTLQYVTTINQRTPLVSENLVVDLG
arabidopsis     SAQTPFRPKALLLPVTKDQSTLQYTTVINQRTPLVPASVVFDLG
```

C-terminal region

```
                    **    * : *.:*:: .: *        .
soybean         GE----YNVRVNSIRITQHSVFP-----LNKIS
lupin           GE----YFIQVNAIRVNKHLVIPTKNPFISPSS
carrot          GEPSVEYFIGVKSIKINSKIVAL------NTSL
arabidopsis     GEKSSEYFIGVTAIQIVEKTVPI------NPTL
```

FIGURE 3: Alignment of amino acid sequences of N- and C-terminal regions in plant 43-kDa-protein-like proteins. The alignment was generated using ClustalX (*18*). Asterisks indicate positions with a single fully conserved residue. Colons indicate positions containing strongly similar residues. Periods indicate positions with weakly similar residues. Soybean, the 43-kDa soybean protein (*19*, GenBank accession number D16107); Lupin, conglutin-γ (*20*, GenBank accession number X65601); Carrot, extracellular dermal glycoprotein (EDGP) (*21*, GenBank accession number D14550); and *Arabidopsis*, *Arabidopsis* EDGP-like protein (GenBank accession number AAC72119).

Table 1: Binding of the 4-kDa Peptide to Fragments of the 43-kDa Protein α Subunit

constructs	4-kDa peptide binding[a]	K_D for 4-kDa peptide ($\times 10^{-8}$ M)	insulin binding
43 kDa	+++	1.80	+
43 kDa α	+++	8.98	ND[b]
43 kDa *β*	−	NC[c]	ND
c1	+++	2.10	ND
c2	++	47.80	ND
c3	++	81.00	ND
c4	+	102.00	ND
c5	+	127.00	ND
c6	+++	13.20	+
c7	−	3890.00	−
c8	−	2420.00	ND
c9	−	NC	ND
c10	−	NC	ND
c11	−	NC	ND
c12	++	52.40	ND
c13	++	58.20	−
c14	+	222.00	+
c15	−	NC	ND

[a] The binding strength of the 4-kDa peptide is indicated as follows: +++, KD < 20×10^{-8} M; ++, 20×10^{-8} < KD < 100×10^{-8} M; +, 100×10^{-8} M < KD < 1000×10^{-8} M; −, KD > 1000×10^{-8} M or no binding. [b] ND = not determined. [c] NC = not calculated because no binding was observed in the sensorgrams.

fusion proteins because these fragments were not well-expressed as soluble His-tag fusion proteins, and the remaining fragments were expressed as His-tag fusion proteins. The purity of all fragments was confirmed by SDS−PAGE followed by staining with Coomassie Blue. All purified proteins were detected as major bands (>90% pure) with the expected sizes (Figure 1B).

The 43-kDa Protein Contains Two Binding Sites for the 4-kDa Peptide. We employed SPR spectroscopy to identify the binding sites for the 4-kDa peptide in the 43-kDa protein. First, the 4-kDa peptide purified from soybean seeds was immobilized in the flow cell of a sensor chip CM5 using an amine coupling according to the method of the SPR instrument supplier. The 4-kDa peptide molecules could be immobilized in the same direction. Hence, the 4-kDa peptide was coupled by only the N-terminal amine group of the molecule because the 4-kDa peptide has no lysine. Various fragments of the 43-kDa protein were used as analytes in the SPR analysis. Solutions of different concentrations were separately passed through the flow cell. Association and dissociation between these fragments and the 4-kDa peptide were detected by measuring increases and decreases in the SPR signals, respectively.

Of these fragments, c13 and c6, containing N- (amino acids 1−43) and C-terminal (amino acids 228−251) regions, respectively, bound the 4-kDa peptide with the highest affinity. The dissociation constants for c13 and c6 were found to be 5.82×10^{-7} and 1.32×10^{-7} M, respectively (Figure 1A and Table 1).

The c3−5 fragments displayed 10-fold lower affinity than that of the C-terminal region (c6). The fragment (129−212th residues) of the α subunit may be involved in the stability of structure. If this region is present predominantly, the binding activity may be decreased.

Before this study, the cysteine-rich region was hypothesized to be the major binding site for the 4-kDa peptide (*12*). In this study, the dissociation constant (K_D) for this region was a little high, 22.2×10^{-7} M (Figure 1A and Table 1). We investigated whether the disulfide bonds in the 43-kDa protein were involved in the interaction between the 43-kDa protein and the 4-kDa peptide. Potential disulfide bonds do not usually form in proteins ectopically expressed in the *E. coli* cytoplasm. Therefore, to express the 43-kDa protein, we used the BL21*trxB* [DE3] strain, which allows the normal disulfide bonds in the expressed protein to form. We recognized that the fragments expressed in *E. coli* should be verified for the correct oxidization of disulfide bridges. However, we could not analyze the pattern of disulfide bridges of the cysteine-rich region because the cysteine-rich region was not hydrolyzed by any methods, such as protease digestion (trypsin, lysylendopeptidase, and pepsin) or chemical hydrolysis.

Komatsu et al. (*12*) reported that the 43-kDa protein has an architecture similar to that of the insulin receptor (IR) and that insulin, gel filtrations (GFs), and the 4-kDa peptide can all interact with the 43-kDa protein. It was proposed that the 43-kDa protein and IR have a similar ligand-binding mechanism. Using alanine-scanning mutagenesis, Whittaker et al. (*15*) investigated the ligand-binding site of the extracellular domain of a type-I IGF receptor. They found that the N-terminal L1 domain, the C-terminal region of the cysteine-rich domain, and the C-terminal domain of the α subunit are critical for ligand binding. Mutation of the cysteine-rich domain did not eliminate the binding activity of the receptor, but the binding activities of the N-terminal L1 and the C-terminal domains of the α subunit were

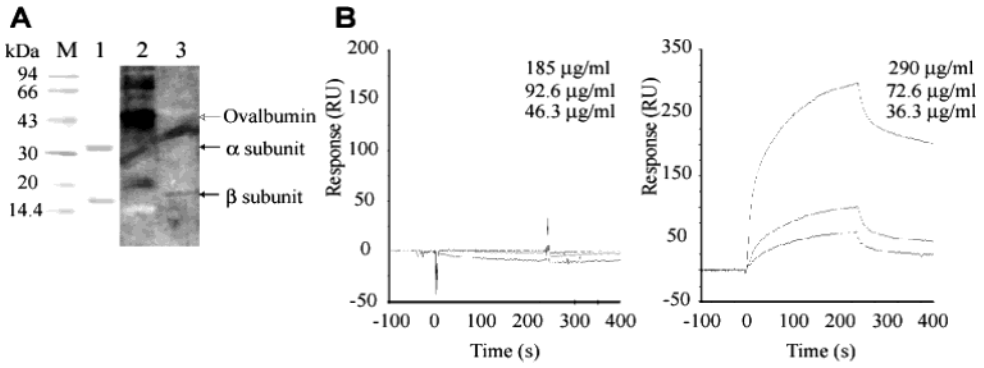

FIGURE 4: Detection of the sugar chains of the 43-kDa protein. (A) Neutral sugar detection. Lane M, molecular markers; lane 1, stained with Ponceau S; lane 2, staining of the sugar chain in lane M; and lane 3, staining of the sugar chain of the 43-kDa protein. Arrows indicate the subunits of the 43-kDa protein (black) and the ovalbumin positive control (white). For details, refer to the Experimental Procedures. (B) Representative sensorgrams showing the interaction of subunits of the 43-kDa protein with Con A. Left, α subunit; and right, *β* subunit. The concentrations of analytes injected into the sensor chip are indicated to the right of the sensorgrams.

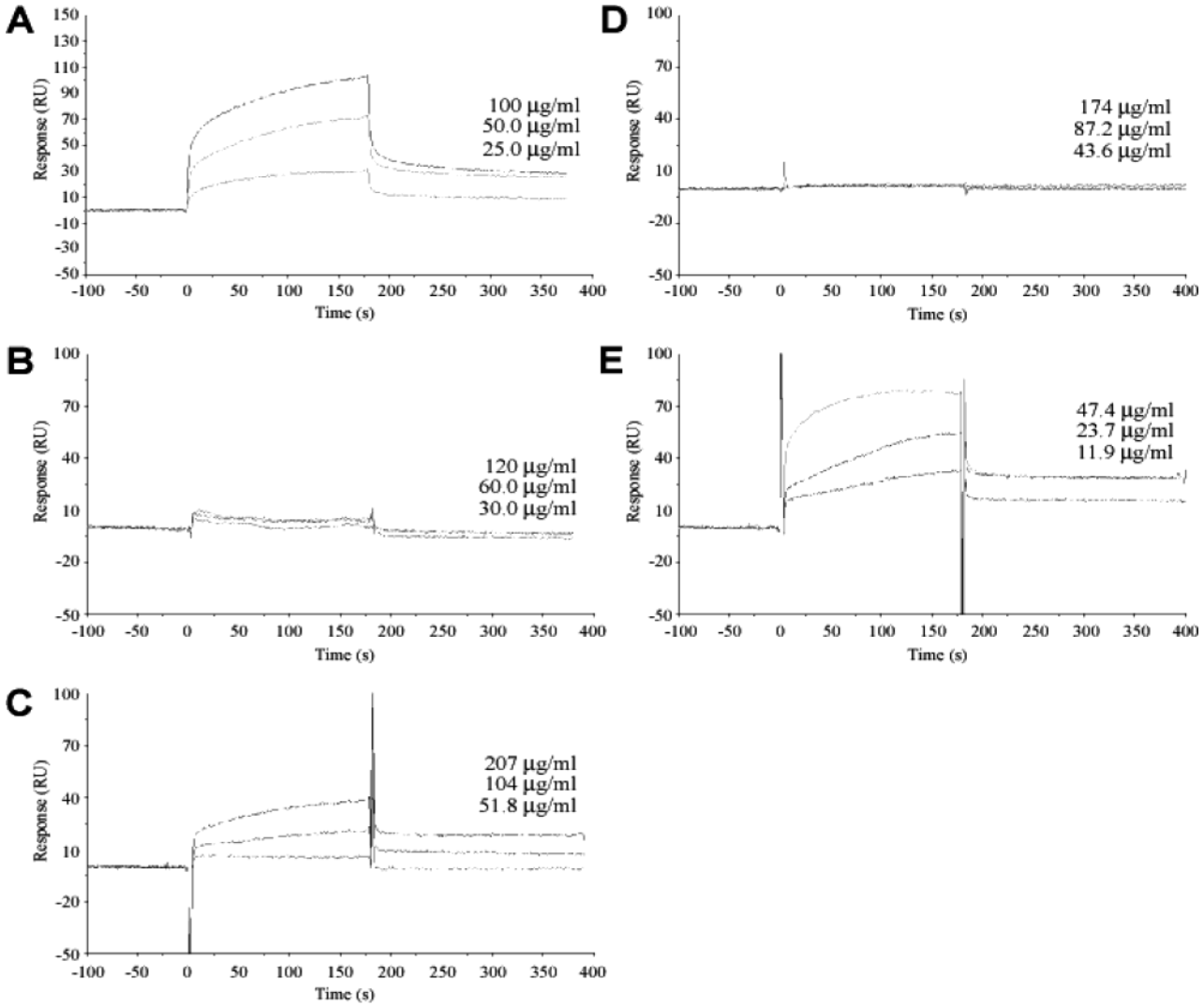

FIGURE 5: Representative sensorgrams showing binding of insulin to expressed fragments of the 43-kDa protein α subunit. (A) Intact 43-kDa protein. (B) c13 (amino acids 1−43). (C) c14 (amino acids 44−128). (D) c7 (amino acids 129−212). (E) c6 (amino acids 212−251). The concentrations of analytes injected into the sensor chip are indicated to the right of the sensorgrams.

extremely sensitive to alanine substitution. They concluded that the cysteine-rich domain assists in ligand binding but is not the major interface.

It was not completely denied that the disulfide pattern was unrelated to the affinity for the 4-kDa peptide. We should conduct further experiments about the cysteine-rich region of the 43-kDa protein because the disulfide pattern of the cysteine-rich region may seriously affect the protein structure.

A multiple sequence alignment of known plant 43-kDa-protein-like proteins reveals that the N-terminal region is highly conserved, suggesting that this region has an important role in the interaction with the 4-kDa peptide (Figure 3).

A

Analyte	Dissociation constants (K_Ds) for N-terminal region (c13) (x 10^{-5} M)	for C-terminal region (c6) (x 10^{-6} M)
Wild-type	0.6	1.3
I25A	35.4	4.8
F28A	8.7	15.9
V29A	55.0	3.2
F31A	57.7	1.5
I33A	11.9	3.4

B

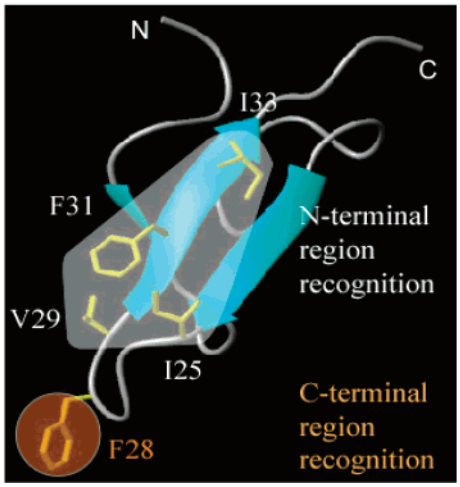

FIGURE 6: Interaction of alanine-substituted mutants of the 4-kDa peptide with the N- and C-terminal regions. (A) Dissociation constants of alanine-substituted mutants for the N- and C-terminal regions obtained from SPR spectroscopy. (B) Functional residues of the 4-kDa peptide for recognition of the N- and C-terminal regions. Putative residues for the N- or C-terminal region recognition were illustrated with mist representation (white, N-terminal region; and orange, C-terminal region).

The C-terminal region of this protein family was less conserved, despite the fact that this region in the soybean protein has high affinity for the 4-kDa peptide (Figure 3). The reasons for the lower level of conservation of this region are unknown.

We have previously reported the amino acid residues of the 4-kDa peptide that are necessary for binding to the 43-kDa protein (*14*). The hydrophobic surface of the 4-kDa peptide is thought to be an interface for binding. SPR spectroscopy indicated that the N- and C-terminal fragments are two major binding fragments in the 4-kDa peptide. These fragments probably form the binding interface in the wide hydrophobic surface of the peptide. This hypothesis should be confirmed by other experiments, such as X-ray crystallography of the 4-kDa peptide and its complex with the 43-kDa protein.

The Sugar Chain of the 43-kDa Protein Is Not Involved in Binding the 4-kDa Peptide. The 43-kDa protein was found to be a glycoprotein containing N-linked sugar chains (*16*). To determine whether these sugar chains are involved in ligand binding, we investigated the carbohydrate chains in the native 43-kDa protein. The 43-kDa protein purified from mature soybean seeds was subjected to SDS−PAGE to separate the α and β subunits, and each subunit was transferred to a PVDF membrane for analysis of the sugar chains. The neutral sugars were biotinylated and then detected using chemiluminescence with streptavidin-conjugated horseradish peroxidase; it was revealed that only the β subunit contains sugar chains (Figure 4A).

The original report that the 43-kDa protein contains sugar chains stated that it is the α subunit that contains a carbohydrate chain, as determined by lectin blotting with Con A (*16*). This result conflicts with our present results. To confirm our results, we examined the interaction between the 43-kDa protein subunits and Con A using SPR spectroscopy. The α and β subunits were prepared using the method of Sato et al. (*17*). No stable binding of the α subunit to Con A was observed using this method (Figure 4B). It is possible that the interaction between the α subunit and Con A is nonspecific. In conclusion, the sugar chains of the 43-kDa protein are not involved in the binding of the 4-kDa peptide to the 43-kDa protein.

The 4-kDa Peptide and Insulin Recognize Similar Regions in the 43-kDa Protein. Although animal insulin interacts with the 43-kDa protein (*12*), it was not known whether insulin and the 4-kDa peptide recognize the same interface. We investigated the insulin-binding region in the 43-kDa protein using four fragments from the α subunit of the 43-kDa protein (c6, c7, c13, and c14) and SPR spectroscopy. Insulin interacted with the fragments containing the cysteine-rich and C-terminal regions of the α subunit (Figures 1 and 5 and Table 1). This is in contrast to the interaction of the 4-kDa peptide with three fragments, the N-terminal, C-terminal, and cysteine-rich regions of the α subunit. These results suggest that the C-terminal and cysteine-rich regions are important for the biological function of the 43-kDa protein.

Because insulin can also activate the protein kinase domain of the β subunit of the 43-kDa protein, the binding site(s) for insulin on the 43-kDa protein might be the same as that for the 4-kDa peptide. We hypothesized that the regions that bind both the 4-kDa peptide and insulin have a critical role in the biological function of the protein. Because the C-terminal region binds both peptides strongly, it is probably the most important region. Although the N-terminal region of the α subunit is one of the major binding sites for the 4-kDa peptide, this region may not be involved in the activation of the 43-kDa protein kinase; it may be involved in the stabilization of the complex of the 43-kDa protein and 4-kDa peptide.

Mapping the Critical Residues of the 4-kDa Peptide that Interact with the N- or C-Terminal Regions of the 43-kDa Protein α *Subunit.* We identified the 4-kDa-peptide-binding regions in the 43-kDa protein. In those regions, N- and C-terminal regions of the α subunit are the most important. In previous work, we demonstrated the residues of the 4-kDa peptide that are involved in the interaction with the 43-kDa protein using alanine-substituted mutants of the 4-kDa peptide (*14*). Thus, to make those critical residues of the 4-kDa peptide correspond to the N- and C-terminal regions of the α subunit, those regions (c6 and c13 fragments) were immobilized to sensor chips using amine coupling, respectively. In the 4-kDa peptide, alanine substitution of Ile-25, Phe-28, Val-29, Phe-31, and Ile-33 exceedingly decreased

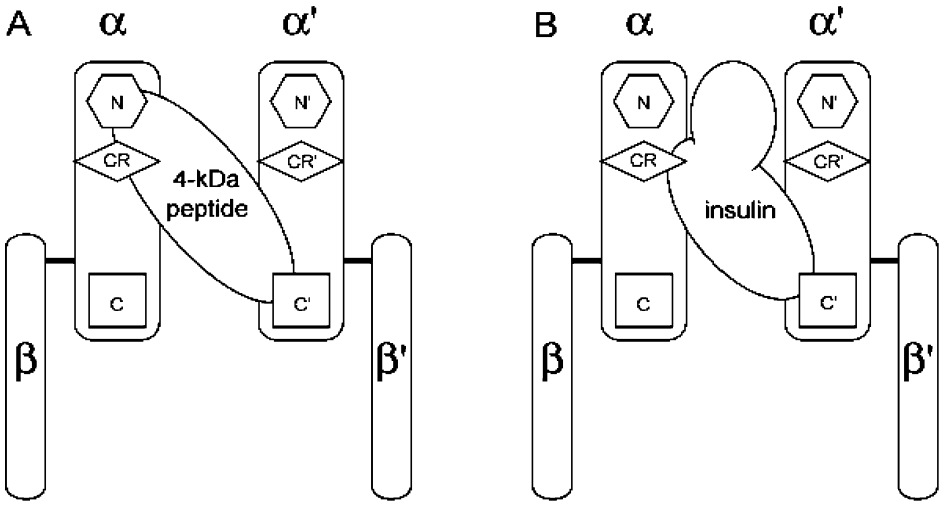

FIGURE 7: Model of the 4-kDa peptide binding to hotspots on the 43-kDa protein. All of binding regions are represented as separated regions. (A) Interaction between the 4-kDa peptide and 43-kDa protein. The C-terminal and cysteine-rich regions (C and CR, respectively) may be involved in the biological activity of the 43-kDa protein. The N-terminal region (N) may be unable to transduce a signal but is involved in potency and selectivity between the 4-kDa peptide and putative surrogate ligands. (B) Interaction between insulin and the 43-kDa protein.

the affinity for the 43-kDa protein, suggesting that those residues were critical for 43-kDa protein binding (*14*). The thioredoxin-tagged wild-type and alanine-substituted mutants of the 4-kDa peptide were produced using the previously reported methods based on a bacterial expression system (*14*) and were subjected to those sensor chips. The results were summarized in Figure 6.

The affinity of the wild-type 4-kDa peptide for the N-terminal region (c13) was 10-fold lower than the result previously reported in Figure 1A and Table 1. This reason was considered to be due to the difference of the direction in immobilization of ligand molecules. Because the protein immobilized onto the sensor chip and the c13 fragment has some lysine residues, it was impossible to control the direction of the molecule immobilized onto the sensor chips. Because each experimental condition differs, it is unavoidable that the dissociation constants were a little different. Although the behavior of the recombinant 4-kDa peptide (wild type) was a little different from that of the wild-type 4-kDa peptide purified from soybean, we guessed that the obtained results in Figure 6 showed the tendency, which seems to be correct.

In the interaction with the C-terminal region of the α subunit of the 43-kDa protein, alanine substitution of Phe-28 of the 4-kDa peptide produced a 3-fold higher K_D than the other mutants, suggesting that Phe-28 was important for the recognition of the C-terminal region of the α subunit. On the other hand, because the interaction of the N-terminal region alanine substitution of Ile-25, Val-29, Phe-31, and Ile-33 resulted in lower affinity, Ile-25, Val-29, Phe-31, and Ile-33 were considered to form the interface to bind the N-terminal region of the α subunit. These results suggested that the N- and C-terminal regions of the α subunit formed the binding spot for the 4-kDa peptide in cooperation.

Model for the Interaction between the 4-kDa Peptide and 43-kDa Protein. In the previous work, we demonstrated that the 4-kDa peptide binds the heterotetramer of the 43-kDa protein, suggesting that the 43-kDa protein had a few binding regions for the 4-kDa peptide (*14*). In this study, we were able to identify at least two independent hotspots (N- and C-terminal regions) on the α subunit. A third region (cysteine-rich region) was also found that appears to lie close to but not overlapping the N-terminal region. The interaction analysis with the alanine-substituted mutants of the 4-kDa peptide showed that the N- and C-terminal regions did not overlap and had different recognition sites for the 4-kDa peptide. A model for the hotspots of the 43-kDa protein is shown as a cartoon in Figure 7. Thus, because both the 4-kDa peptide and insulin can interact with the 43-kDa protein, the C-terminal and cysteine-rich regions are probably important for activation of the 43-kDa protein. However, because of difference of affinity for the 4-kDa peptide, the C-terminal region should be the most important for biological function, although it is not clear from the present study whether binding of one or two ligand molecules is required for activation of the 43-kDa protein. The role of the N-terminal regions, other than to provide additional contacts and increase binding affinity, is less clear. However, one possibility is that the N-terminal region may mark the hotspot on the 43-kDa protein that is responsible for the 4-kDa peptide potency and selectivity based on the fact that the N-terminal region is specific to the 4-kDa peptide and does not bind to insulin.

In this model of activation of the 43-kDa protein, the 43-kDa protein exists as a heterotetramer where the 4-kDa peptide is not required to induce dimerization but induces its agonist effect by cross-linking separate binding regions in the two adjacent subunits of the heterotetramer. One possibility could be that activation of the 43-kDa protein requires binding of two ligand molecules. However, the gel-filtration analysis did not support this hypothesis (*14*). It is intriguing to ask whether the 43-kDa protein is activated by one or two ligands. Studies to elucidate the molecular mechanism of activation of the 43-kDa protein by alternative methods, such as X-ray crystallography, are currently ongoing.

REFERENCES

1. Niall, H. D. (1982) The evolution of peptide hormones, *Annu. Rev. Physiol. 44*, 615−624.
2. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and Walter, P. (2002) *Molecular Biology of the Cell*, 4th ed., Garland Science, New York.

3. van der Geer, P., Hunter, T., and Lindberg, R. A. (1994) Receptor protein-tyrosine kinases and their signal transduction pathways, *Annu. Rev. Cell Biol. 10*, 251−337.
4. Kende, H., and Zeevaart, J. A. D. (1997) The five "classical" plant hormones, *Plant Cell 9*, 1197−1210.
5. Pearce, G., Strydom, D., Johnson, S., and Ryan, C. A. (1991) A polypeptide from tomato leaves induces wound-inducible proteinase inhibitor proteins, *Science 253*, 895−898.
6. Ryan, C. A., Pearce, G., Scheer, J., and Moura, D. S. (2002) Polypeptide hormones, *Plant Cell 14*, S251−S264.
7. Watanabe, Y., Barbashov, S. F., Komatsu, S., Hemming, A. M., Miyagi, M., Tsunasawa, S., and Hirano, H. (1994) A peptide that stimulates phosphorylation of the plant insulin-binding protein: Isolation, primary structure, and cDNA cloning, *Eur. J. Biochem. 224*, 167−172.
8. Yamazaki, T., Takaoka, M., Katoh, E., Hanada, K., Sakita, M., Sakata, K., Nishiuchi, Y., and Hirano, H. (2003) A possible physiological function and the tertiary structure of a 4-kDa peptide in legumes, *Eur. J. Biochem. 270*, 1269−1276.
9. McDonald, N. Q., and Hendrickson, W. A. (1993) A structural superfamily of growth factors containing a cystine-knot motif, *Cell 73*, 421−424.
10. Nishizawa, N. K., Mori, S., Watanabe, Y., and Hirano, H. (1994) Ultrastructural localization of the basic 7S globulin in soybean (*Glycine max*) cotyledons, *Plant Cell Physiol. 35*, 1079−1085.
11. Komatsu, S., and Hirano, H. (1991) Plant basic 7S globulin-like proteins have insulin and insulin-like growth factor binding activity, *FEBS Lett. 294*, 210−212.
12. Komatsu, S., Koshio, O., and Hirano, H. (1994) Protein kinase activity and insulin-binding activity in plant basic 7S globulin, *Biosci. Biotechnol. Biochem. 58*, 1705−1706.
13. Yamauchi, F., Sato, K., and Yamagishi, T. (1984) Isolation and partial characterization of a salt-extractable globulin from soybean seeds, *Agric. Biol. Chem. 48*, 645−650.
14. Hanada, K., Nishiuchi, Y., and Hirano, H. (2003) Amino acid residues on the surface of soybean 4-kDa peptide involved in the interaction with its binding protein, *Eur. J. Biochem. 270*, 2583−2592.
15. Whittaker, J., Groth, A. V., Mynarcik, D. C., Pluzek, L., Gadsbøll, V. L., and Whittaker, L. J. (2001) Alanine scanning mutagenesis of a type 1 insulin-like growth factor receptor ligand binding site, *J. Biol. Chem. 276*, 43980−43986.
16. Hirano, H., Kagawa, H., and Okubo, K. (1992) Characterization of proteins released from legume seeds in hot water, *Phytochemistry 31*, 731−735.
17. Sato, K., Yamagishi, T., Kamata, Y., and Yamauchi, F. (1987) Subunit structure and immunological properties of a basic 7S globulin from soybean seeds, *Phytochemistry 26*, 903−908.
18. Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., and Higgins, D. G. (1997) The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools, *Nucleic Acids Res. 24*, 4876−4882.
19. Watanabe, Y., and Hirano, H. (1989) Sequence of a cDNA encoding soybean basic 7S globulin, *Nucleic Acids Res. 17*, 8868.
20. Kolivas, S., and Gayler, K. R. (1993) Structure of the cDNA coding for conglutin γ, a sulphur-rich protein from *Lupinus angustifolius*, *Plant Mol. Biol. 21*, 397−401.
21. Satoh, S., Sturm, A., Fujii, T., and Chrispeels, M. J. (1992) cDNA cloning of an extracellular dermal glycoprotein of carrot and its expression in response to wounding, *Planta 188*, 432−438.

BI049587A