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Amino acid substitutions from an indispensable disulfide bond affect $P2X_2$ receptor activation

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Abstract

The roles of six amino acid residues downward from an extracellular disulfide bond involving Cys^{224} in rat $P2X_2$ receptor were examined. When Cys^{224} or Pro^{225} was replaced with alanine, the responsiveness to ATP was lost. When Ile^{226} was replaced with other hydrophobic amino acids, the responsiveness to ATP was reduced or abolished. When Phe^{227} was replaced with leucine or isoleucine, the responsiveness to ATP was abolished. The responsiveness to ATP was moderately decreased with the alanine-substitution for Arg^{228} and it was markedly decreased with the alanine-substitution for Leu^{229}. As for the alanine-substitution for Gly^{230}, the sensitivity was changed, but the maximal response to ATP was not reduced. The results suggested that a precise structure is required for amino acid residues close to the disulfide bond and, in general, the amino acid residues at odd number positions and those closer to the disulfide bond are more influential to the ATP responsiveness.

Keywords: P2X receptor; ATP; Site-directed mutagenesis; *Xenopus* oocyte; Membrane current

1. Introduction

P2X receptors are ion channel-forming membrane proteins that are activated by extracellular ATP (see reviews, Khakh, 2001; North, 2002). One functional ion channel is presumably formed by three homogenous subunits. Each subunit has two transmembrane regions (TM1 and TM2) and a long extracellular loop (E1) between them. Basic amino acid residues near the outer mouth of the channel pore formed by TM1 and TM2 appear to serve as a part of the binding pocket of ATP molecules (Ennion et al., 2000; Jiang et al., 2000). In addition to these basic residues, amino acid residues in E1 apart from the channel pore have been shown to affect the channel activation by ATP. $P2X_2$ receptor did not respond to ATP when Gly^{247} in E1 was replaced with alanine (Nakazawa and Ohno, 1999). The sensitivity to ATP was reduced when Gly^{248} was replaced with valine and the responsiveness was lost when the residue was replaced with leucine (Nakazawa et al., 2002). The replacement of Trp^{256} with other amino acid residues except for tyrosine also resulted in the loss of responsiveness (Nakazawa et al., 2002). These results suggest that the structure around these positions should be precisely maintained for the channel activation by ATP. Recently, the pairs of cysteines that form disulfide bonds have been identified for $P2X_1$ (Ennion and Evans, 2002) and $P2X_2$ (Clyne et al., 2002) receptors. Among these cysteines, the structure formed by Cys^{224} through a disulfide bond with Cys^{214} appears to be critical because the replacement of this residue with alanine was non-functional (Clyne et al., 2002). Cys^{227} in $P2X_1$, which corresponds to Cys^{224} in $P2X_2$ (see Fig. 1), may also play an indispensable role in the formation of such a structure because its alanine substitution resulted in about 50-fold reduction in the sensitivity to ATP (Ennion and Evans, 2002). In the present study, we examined the roles of amino acid residues following Cys^{224} in $P2X_2$ receptor to understand

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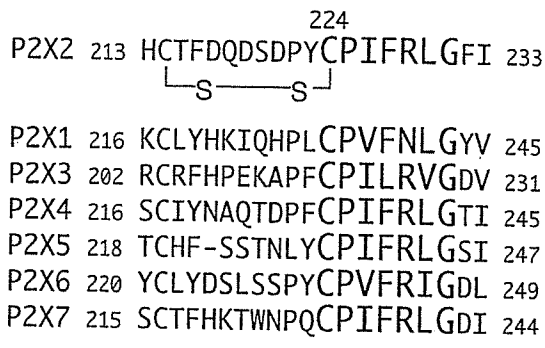

Fig. 1. Amino acid sequence near Cys^{224} in the extracellular loop of $P2X_2$ receptor and corresponding sequences of P2X receptor subclasses. Amino acid residues targeted for site-directed mutagenesis and those in the equal positions were shown as the larger characters. Cys^{224} forms a disulfide bond with Cys^{214}.

structural requirements for the channel activation in this region.

2. Materials and methods

2.1. Oocyte expression and membrane current measurements

The expression of cloned and mutant $P2X_2$ receptor and the recordings of ionic current through the channels were performed according to our previous report (Nakazawa et al., 1998). Briefly, $P2X_2$ receptor mutants were constructed from the cloned $P2X_2$ receptor (Brake et al., 1994) by site-directed mutagenesis. Amino acid residues targeted for mutagenesis were shown in Fig. 1. The wild-type and the mutant channels were expressed in *Xenopus* oocytes for a 4-day incubation at 18 °C and the oocytes were served for membrane current measurements. Oocytes were bathed in ND96 solution containing (in mM) NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 5 (pH 7.5 with NaOH). ATP (adenosine 5′ -triphosphate disodium salt; Sigma, St. Louis, MO, USA) was applied by superfusion for about 6 s with a regular interval of 1 min.

2.2. Immunoblotting analysis

The expression of channel protein was confirmed by immunoblotting analysis. Crude membrane fractions were prepared from oocytes (20 oocytes for the wild-type channel and each mutant) according to Newbolt et al. (1998) with minor modifications. Oocytes were suspended in a 50 × stock of a protease and phosphatase inhibitor cocktail (Sigma, general use; 1 bottle for 100 ml effective solution) diluted in a buffer containing 20 mM Tris–HCl, 2 mM EDTA (disodium salt), 0.5 mM EGTA and 320 mM sucrose by pipetting. The homogenate was horizontally shaken at 100 rpm for 15 min at 4 °C and then centrifuged at 14,000 rpm for 2 min. The supernatants were analyzed by SDS-PAGE gel electrophoresis and immunoblotting. By using $P2X_2$ receptor antibodies (Oncogene, Boston, MA, USA) and anti-rabbit Ig, horseradish peroxidase-linked whole antibody (from donkey; Amersham, Little Chalfont, England), correct channel expression was detected as a 65-kDa band.

2.3. Data analysis

Parameters for ATP sensitivity (EC_{50}, pD_2 and Hill coefficient) were obtained from current responses using the following equation:

$$E = E_{max}A^n/[A^n + (EC_{50})^n], \quad (1)$$

where E is an effect (current response), E_{max} is an maximal response, A is ATP concentration, EC_{50} is concentration required for a half-maximal effect and n is a Hill coefficient (slope factor). When EC_{50} and n were calculated from ATP concentrations used and obtained current responses, Eq. (1) was transformed to:

$$\log[E/(E_{max} - E)] = n(\log A - \log EC_{50}), \quad (2)$$

and linear regression was made using Microsoft® Excel X. pD_2 values were negative logarithm of EC_{50} values. When experimental data were fitted by a two binding-site model, the fraction of one binding-site (f) and that of the other binding-site ($1 - f$) were introduced. The effect mediated through each binding-site was calculated from Eq. (1) and the fraction (f or $1 - f$), and the sum of these effects was obtained.

3. Results

3.1. Amino acid substitutions and ATP responsiveness

Cys^{224} and neighboring Pro^{225} are completely conserved among seven subclasses of P2X receptors (Fig. 1; Soto et al., 1997). Fig. 2 shows concentration–response relationship for ATP-evoked current recorded from oocytes expressing the wild-type $P2X_2$ receptor and the Cys^{224}- or Pro^{225}-to-alanine-substituted mutants (C224A or P225A, respectively). C224A exhibited no current response to ATP, as has been reported by Clyne et al. (2002). P225A also failed to respond to ATP. To examine the effects of amino acid residues succeeding to Pro^{225}, we first constructed deletion mutants. When three residues next to Pro^{225} (Ile^{226}, Phe^{227} and Arg^{228}) were deleted, the responsiveness to ATP was lost. Deletion of the former two or even Ile^{226} alone also resulted in the loss of the responsiveness. The preliminary results suggest that these amino acid residues also appeared to be essential. Thus, instead of deletion, we introduced amino acid substitutions to these residues in a one-by-one manner.

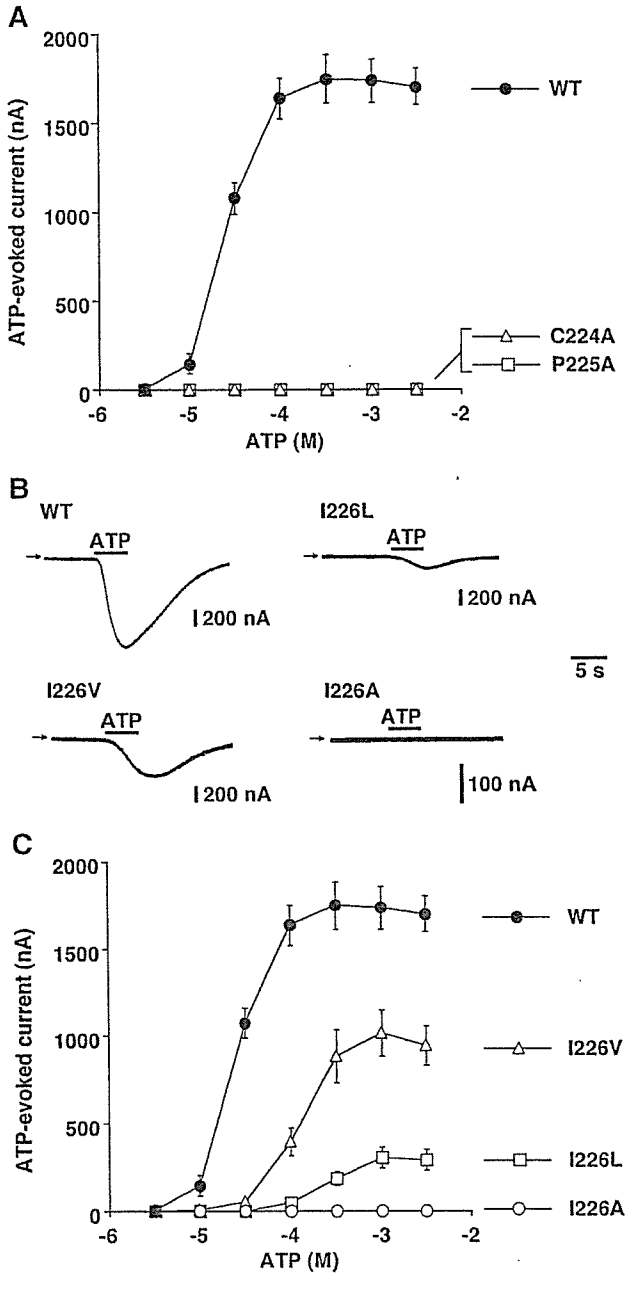

Fig. 2. (A) Disappearance of current responses to ATP by the replacement of Cys^{224} or Pro^{225} with alanine (C224A or P225A). Peak amplitude of currents activated by ATP at −50 mV was plotted against ATP concentrations. Each symbol and bar represent mean and S.E. obtained from five to eight oocytes. In contrast to current responses in oocytes expressing the wild-type channel (WT), no response was observed in those expressing the mutant channels. (B) Currents activated by 30 μM ATP in oocytes expressing the wild-type (WT) and Ile^{226}-to-leucine (I226L), -valine (I226V) and -alanine (I226A)-substituted mutant channels. The oocytes were held at −50 mV. Arrows indicate zero current levels. (C) Concentration–response relationship for the wild-type and Ile^{226}-replaced channels. The current responses were obtained as shown in B. The concentration–response relationship for the wild-type channel (WT) was also shown for comparison. Each symbol and bar represent mean and S.E. obtained from five to eight oocytes.

Ile^{226} was replaced with hydrophobic amino acid residues because this position was occupied by isoleucine or valine in seven P2X receptor subclasses (Fig. 1). Fig. 2B shows current responses to 100 μM ATP in oocytes expressing the wild-type and the mutant channels. When substituted with leucine, which has a volume similar to that of isoleucine (Chothia, 1975), the ATP-evoked current was markedly reduced (Fig. 2B,C; I226L). When substituted with valine, a smaller hydrophobic residue, the reduction of the current

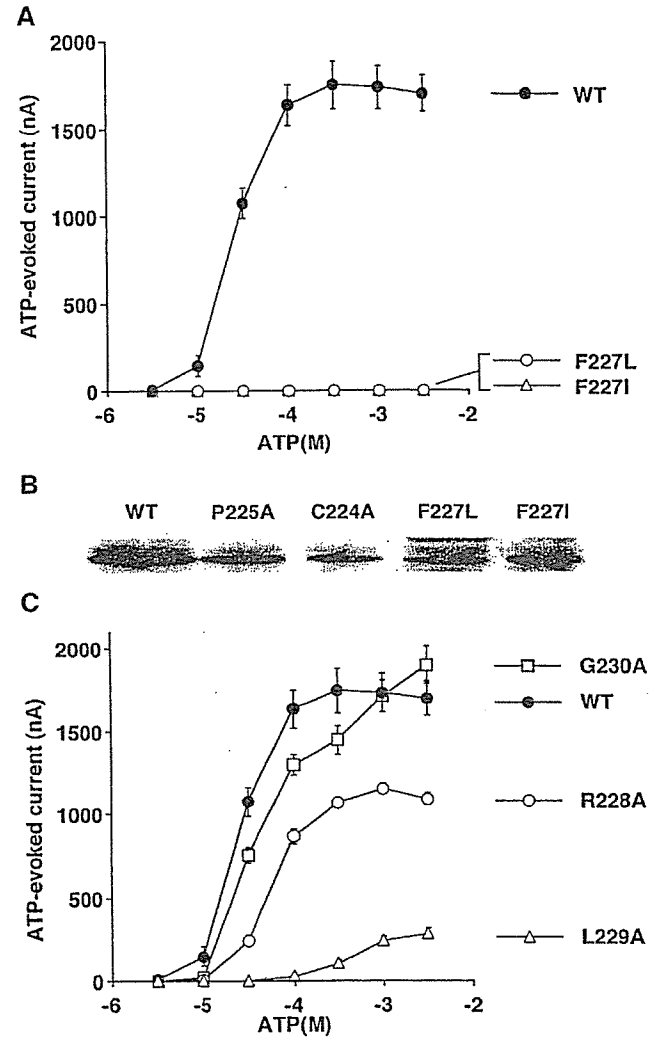

Fig. 3. (A) Disappearance of current responses to ATP by the replacement of Phe^{227} with leucine (F227L) or isoleucine (F227I). Data obtained from five to eight oocytes were shown as in Fig. 2A. (B) Immunoblotting analysis using anti-$P2X_2$ receptor antibody. A section corresponding to $P2X_2$ receptor protein (about 65 kDa) was shown to compare the expression in membrane fractions prepared from the oocytes injected with the wild-type channel (WT) and four non-ATP responsive mutants shown in Fig. 2A (C224A and P225A) and A of this figure (F227L and F227I). (C) Concentration–response relationship for the wild-type and Arg^{228}-(R228A), Leu^{229}- (L229A) and Gly^{230}-to-alanine-substituted mutants. The concentration–response relationship for the wild-type channel (WT) was also shown for comparison. Data obtained from five to eight oocytes were shown as in Fig. 2A.

was less remarkable than in the case of the leucine-substitution (Fig. 2B,C; I226V). When substituted with alanine, a hydrophobic residue smaller than valine, the current responsiveness to ATP was abolished (Fig. 2B,C; I226A).

Phe^{227}, a residue succeeding to Ile^{226}, is conserved among six of seven P2X receptor subclasses (Fig. 1). In the remaining subclass ($P2X_3$), leucine is present instead of phenylalanine. When Phe^{227} was replaced with leucine, the current response to ATP disappeared (Fig. 3A; F227L). The current response has also disappeared when replaced with isoleucine (Fig. 3A; F227I). The disappearance of the current response may not be due to non-expression of receptor protein because the protein expression of F227L or F227I was confirmed by immunoblotting analysis (Fig. 3B). The protein expression was also confirmed for the C224A, P225A (Fig. 3B) or I226A (not shown).

Three residues succeeding to Phe^{227} were alanine-substituted (Fig. 3C). The current response to ATP was reduced to about 60% when Arg^{228} was replaced with alanine (R228A). The current response was, however, much reduced (to about 20%) when Leu^{229} was replaced with alanine (L229A). As for the replacement of Gly^{230}, the maximal current response was comparable to that with the wild-type channel (G230A).

3.2. Sensitivities to ATP

To compare the sensitivity to ATP, the concentration–response data were normalized to the maximal response (Fig. 4). Theoretical curves were fitted to the normalized data (see Section 2), and EC_{50} values and Hill coefficients were determined. For the wild-type channel, the EC_{50} value was 29 μM and the Hill coefficient was 2.3. Ile^{226}-, Arg^{228}- and Leu^{229}-substituted mutants exhibited lower sensitivities to ATP than the wild-type channel did (Fig. 4A–C), and the order of the sensitivities was R228A>I226V ≈ I226L>L229A. Hill coefficients for these mutants (varying from 1.7 to 2.6) were similar to that for the wild-type channel. When the curve fitting was applied to the data with G230A, the Hill coefficient as well as the ATP-sensitivity was lower than those for the wild-type channel (Fig. 4D, solid curve). The data points in Fig. 4D were, however, not well fitted to the solid curve. In fact, a regression coefficient of the fitting for the G230A

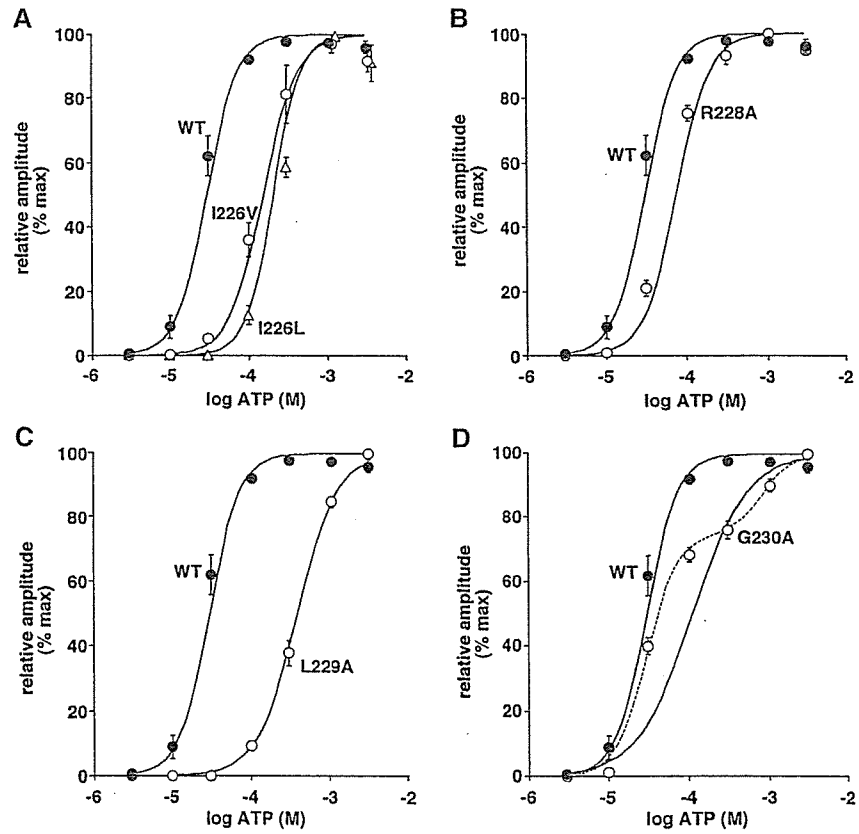

Fig. 4. (A–C) Curve-fittings to concentration–response data for the wild-type and the mutant channels. Current responses to ATP were normalized to maximal responses in individual oocytes and mean values were plotted against ATP concentrations. A curve was fitted to data assuming a homogenous binding-site for each channel type as shown in Section 2. Bars are S.E. Parameters calculated and used for fittings are: EC_{50} (in μM); 29 (WT), 151 (I226V), 202 (I226L), 72 (R228A), 379 (L229A), 108 (G230A); Hill coefficient; 2.3 (WT), 2.0 (I226V), 2.6 (I226L), 2.1 (R228A), 1.7 (L229A), 1.3 (G230A). (D) Curve-fittings to concentration–response data for G230A mutants. The data and were shown and the solid curve with an EC_{50} value of 108 μM and a Hill coefficient of 1.3 was fitted to the data as in A–C. For the broken curve, it was constructed assuming 75% of binding sites are equivalent to the wild-type receptor and the remaining 25% of binding sites have a lower affinity (EC_{50}; 800 μM) and the same slope (Hill coefficient; 2.3).

($r=0.91$) was markedly smaller than those for the remaining channels (0.98–1.00). The poor fitting suggests that this simple theoretical fitting was not available for G230A. Thus, a fitting was made assuming two independent binding sites (Fig. 4D, broken curve). A curve could be fitted with the data when assuming that 75% of the current response is mediated through a binding site having the same EC_{50} and Hill coefficient as the wild-type channel does, and the remaining 25% is mediated through another binding site having the same Hill coefficient but a larger EC_{50} value (800 μM).

3.3. ATP responsiveness and amino acid positions

In Fig. 4, we used the mean values for the curve fittings. Similar curve fittings were applied to data obtained from individual oocytes, and sensitivities to ATP were determined. pD_2 values obtained in this manner as well as the maximal current responses were plotted for the wild-type and the mutant channels in Fig. 5. For the channels with which the current responses to ATP were observed, a clear correlation was found between the maximal current amplitude and the ATP sensitivity; the channels having exhibited smaller maximal current amplitude exhibited lower sensitivity to ATP. In the amino acid sequence beginning from Ile^{226} to Gly^{230}, the substitution of an amino acid residue at an odd number position resulted in larger changes than that at an even number position. For example, the substitution of Phe^{227} resulted in the loss of ATP responsiveness, whereas the substitution of Ile^{226} or Arg^{228} did not lose the responsiveness in most cases. Similarly, the substitution of Leu^{229} resulted in large decreases in both the maximal amplitude and the ATP sensitivity, but the substitution of Arg^{228} or Gly^{230} resulted in relatively small decreases in these indexes. Within the odd or even number positions, the substitution of an amino acid residue closer to the disulfide bond involving Cys^{224} resulted in larger changes in the ATP responsiveness. The substitutions of Phe^{227} resulted in the loss of the

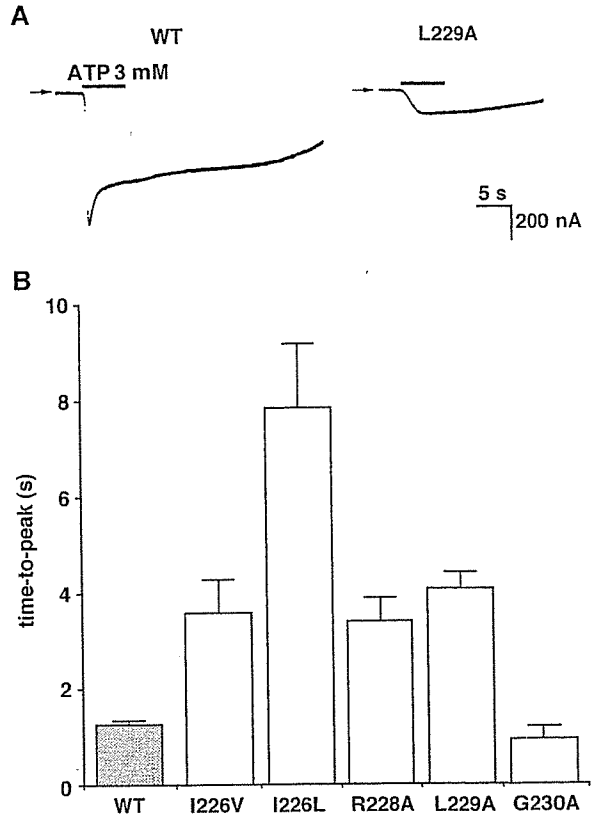

Fig. 6. Activation time course of ATP-evoked current through the wild-type and mutant channels. (A) Currents activated by 3 mM ATP in oocytes expressing the wild-type (WT) and L229A mutant channels. The oocytes were held at −50 mV. Arrows indicate zero current levels. (B) Comparison of time required for peak current evoked by 3 mM ATP ("time-to-peak"). Each column and bar represent mean and S.E. obtained from five to eight oocytes.

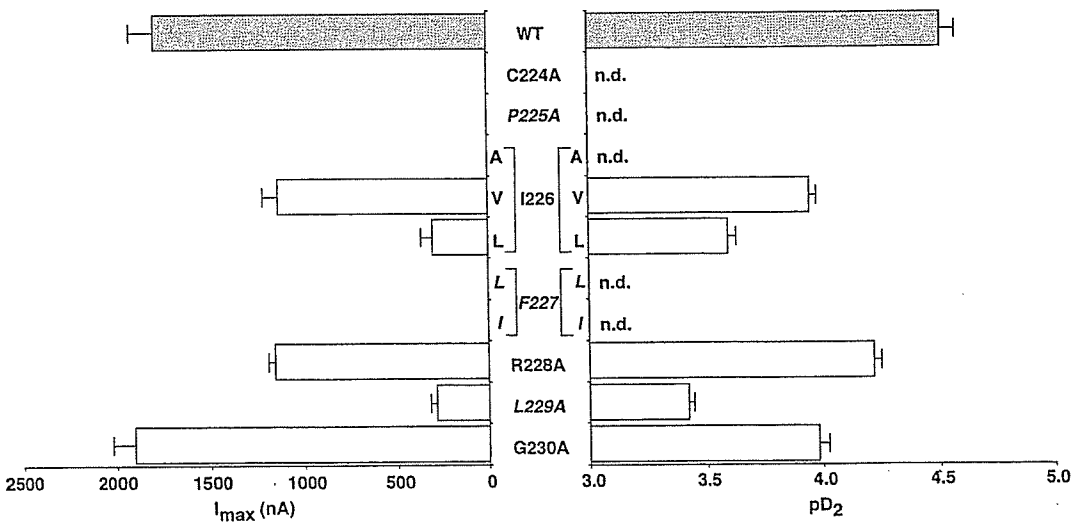

Fig. 5. Comparison of the maximal current amplitude (I_{max}) and the sensitivity to ATP (pD_2) among the wild-type and the mutant channels. The parameters were calculated from each oocytes and mean values were shown for each channel type. Bars are S.E. n.d.: not determined because of the loss of the ATP responsiveness.

responsiveness, whereas the substitution of Leu^{229} resulted in large decreases in the indexes for the responsiveness, but not in the loss of responsiveness. As for the amino acid substitutions at the even number positions, the order of the maximal current responses was Gly^{230}>Arg^{228}>Ile^{226} though the ATP sensitivities were comparable between Arg^{228} and Gly^{230}.

3.4. Activation kinetics

When ATP was applied, activation kinetics was generally faster for the wild-type channel than that for the low responsiveness mutants such as I226V or L229A. To compare the activation kinetics quantitatively, we measured the time required for peak current amplitude when activated by 3 mM ATP ("time-to-peak"; Fig. 6). The time-to-peak was <2 s for the wild-type channel, whereas it was >3 s for the low responsiveness mutants (I226V, I226L, R228A and L229A). The time-to-peak for G230V was similar to that for the wild-type channel.

4. Discussion

We have examined the effects of amino acid substitutions downward from Cys^{224} in the extracellular loop of $P2X_2$ receptor. When Cys^{224} was replaced with alanine, the responsiveness to ATP was lost (Fig. 2A), suggesting that the disulfide bond involving this residue is indispensable for the responsiveness as has been reported by Clyne et al. (2002). The alanine-substitution of Pro^{225} also resulted in the loss of the ATP responsiveness (Fig. 2A). Proline residues are able to disturb the formation of rigid structures such as α-helixes or β-sheets. This ability of Pro^{225} may provide distortion necessary for Cys^{224} to contribute to the formation of the disulfide bond.

As for the substitutions of Ile^{226}, the current response to ATP largely remained when substituted with valine (Fig. 2B,C). In contrast, the substitution with leucine resulted in a marked decrease in the current response (Fig. 2B,C). These results are puzzling because, among hydrophobic residues, leucine is similar to isoleucine in size but valine is smaller than these residues (Chothia, 1975). Interestingly, five of seven P2X receptor subclasses possess an isoleucine residue in this position, and the remaining two subclasses possess a valine residue (Fig. 1). Some common property between isoleucine and valine is necessary to maintain structures proper for the channel activation.

The substitutions of Phe^{227} with hydrophobic amino acid residues (leucine and isoleucine) resulted in the loss of the ATP responsiveness (Fig. 2A). Among P2X receptor subclasses, $P2X_3$ alone has leucine and all the remaining six subclasses have phenylalanine in this position. When Phe^{227} is replaced with leucine, five sequential amino acid residues beginning from Cys^{224} in $P2X_2$ receptor (cysteine–proline–isoleucine–leucine–arginine) completely accord with the residues at corresponding positions in $P2X_3$ receptors (Fig. 1). In spite of this fact, the replacement resulted in the loss of the channel function.

The results obtained from the alanine-substitution of Arg^{228}, Leu^{229} and Gly^{230} showed that a distinct reduction in the ATP responsiveness was found for L229A, but not for R228A or G230A (Fig. 3C). By combining these results and the results obtained from the substitutions of Ile^{226} and Phe^{227}, it appears that the replacement of the residues at odd number positions more dramatically reduces the ATP responsiveness than that at even number positions (Fig. 5). Freist et al. (1998) have pointed out that a sequence stretch of the positions 170–330 in the extracellular loop of P2X receptor proteins exhibits similarities with the catalytic domains of class II aminoacyl-tRNA synthetases as shown by secondary structure predictions and sequence alignments. In their prediction, the region involving the above-mentioned amino acid residues participates in the formation of β-sheets. If these residues are involved in the β-sheets, the residues aligning in one side (Phe^{227} and Leu^{229}) may be more influential on the channel function of P2X receptor than those in the other side (Ile^{226}, Arg^{228} and Gly^{230}).

The Hill coefficient of the wild-type $P2X_2$ receptor was about 2 (Fig. 4), which is in common with those in other reports (e.g., Nakazawa et al., 1991; Nakazawa, 1994). The value of 2 may indicate that the channel activation requires two ATP molecules (Tallarida and Jacob, 1979) though Bean (1990) has shown that the activation requires three ATP molecules at lower concentrations. The Hill coefficient of about 2 was not affected by the substitutions of the amino acid residues tested except for the alanine-substitution of Gly^{230} (Fig. 4). G230A exhibited a Hill coefficient of 1.3 when assuming homogenous binding sites (Fig. 4D, solid curve), but a better fitting could be obtained when the second binding sites of a lower affinity (Fig. 4D, broken curve). For the latter fitting, a Hill coefficient of about 2 was adopted for both the higher and the lower affinity sites. Thus, the introduction of alanine into the position 230 may not affect the number of ATP molecules for the channel activation, but may affect some process involved in the channel activation.

The present study and our previous studies (Nakazawa and Ohno, 1999; Nakazawa et al., 2002) have shown that small changes in a sequence from about the position 220 to about the position 260 in the extracellular loop can result in the loss of the ATP responsiveness. Jiang et al. (2000) also reported that alanine-substitution of Asn^{202} or Asp^{261} resulted in the loss of the ATP responsiveness. In addition, Buell et al. (1996) reported that $P2X_4$ receptor, which is insensitive to two-subclass selective purinoceptor antagonists (pyridoxal-5-phosphate-6-azophenyl-2′,4′-disulfonic acid and pyridoxal 5-phosphate), restored sensitivities to these antagonists when Glu^{249} was replaced with lysine. These results suggest that this extracellular amino acid sequence may directly contribute to some indispensable process between the recognition of ATP molecules and the

channel opening. Ennion et al. (2000) suggested that basic amino acid residues close to the channel pore (Lys^{68}, Lys^{70}, Arg^{292} and Arg^{309}) serve to recognize ATP molecules in $P2X_1$ receptor and, thus, the ATP binding pocket may form close to the outer mouth of the channel pore. Similar results were also obtained for $P2X_2$ receptor (Jiang et al., 2000). The extracellular amino acid sequence described above (the positions 224–230) is not located between this possible binding pocket and the channel pore. If this sequence contributes to some indispensable process between the recognition of ATP molecules and the channel opening, it is desired that the sequence is spatially positioned close to the "activation" link between the binding pocket and the channel pore. This view may be supported by the slower activation process observed in the low responsiveness mutants (Fig. 6). It is possible that the extracellular loop is "packed" densely enough for the sequence to reach the "activation" link.

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Synthesis of C_{60} derivatives for photoaffinity labeling

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Abstract—In order to study the interaction of fullerenes with biological molecules, a novel photoaffinity labeling agent derived from C_{60} was designed and synthesized. As photosensitive functional groups, azide group, and aziridine group are utilized. A convenient synthetic route via fulleropyrrolidine **2** was employed to obtain compounds labeling agents **5** and **9**.

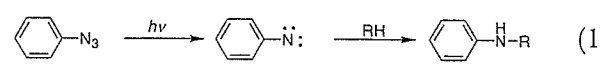
The biological activities of fullerenes have attracted considerable attention due to their potential medicinal applications.[1–3] Their novel and unexploited properties stem from their bulky hydrophobic shape and their photosensitivity[4–7] and radical-generating[8–11]/-quenching[12,13] activities enabled by highly conjugated π-electron system.

As a most remarkable activity, direct inhibition of enzymes by C_{60} has been reported. The first example, HIV-1 protease inhibition by a water soluble fullerene derivative, was reported in 1993[14–16] by Wudl, Wilkins, et al. Independently, Toniollo et al. has reported C_{60}-peptide conjugates and identified activity of these compounds against HIV-1 protease and chemotatic activity against human monocytes.[17] Separately, we have developed new procedures for solubilizing C_{60} in water[18] and assayed unfunctionalized C_{60} for direct enzymatic inhibtion. These studies led to the discovery that aqueous solutions of C_{60} inhibit glutathione-*S*-transferase (GST).[19]

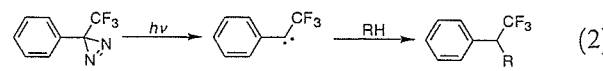
The ability of C_{60}, which is large (7 Å id) hydrophobic molecules, to bind to biological compounds, was initially surprising and several groups have attempted to identify and calculate the binding sites. Based on a computer simulated docking study, Wudl, Wilkins, et al. speculated that the C_{60} core was enclosed in the cylindrical active site, which consists primarily of hydrophobic amino acid residues, of HIV-1 protease. In our own work, we calculated that C_{60} binds to GST at a cleft between two subunits of the enzyme, although the specific residues, which make up the active site are unclear.[20]

In order to clarify the more detailed binding site of C_{60}, two solutions are possible as follows. One is to isolate pure enzyme–fullerene complex and determine the structure by NMR or crystallographic methods. Another potential method for identifying the active site area is photoaffinity labeling, which is particularly useful for identifying the active site in solution under physiological conditions.

We now report the design and synthesis of the first C_{60}-derived photoaffinity labeling reagents. Our synthetic route to photoaffinity reagents **5** and **9** provide a concise, flexible route to fullerenes functionalized with photoreactive pendant groups such as phenylazide and phenyldiazirine, which generate aryl nitrene and aryl carbene, respectively (Eqs. 1 and 2).[21]



(1)



(2)

In order to develop an efficient and flexible synthetic method, which would allow the late-stage introduction of a variety of photoaffinity labels, we chose to utilize dimethylfulleropyrrolidine (**2**). This C_{60} derivative

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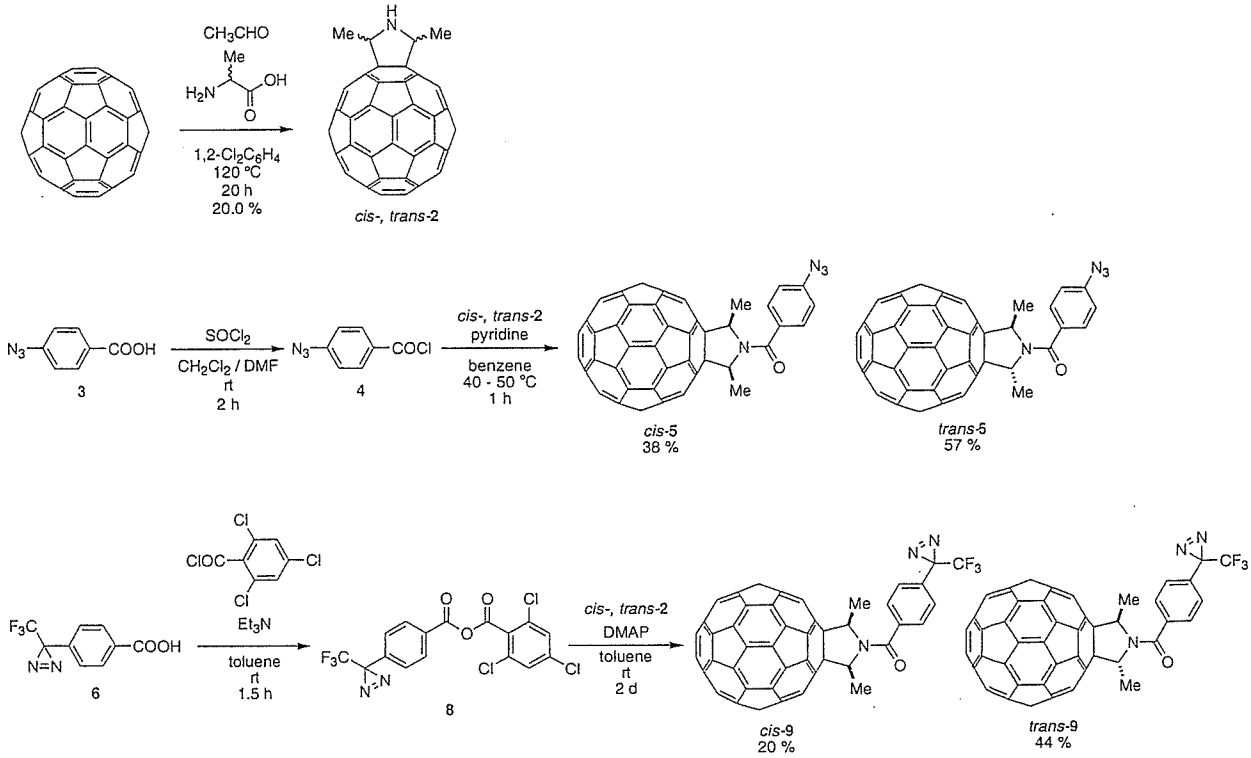

Scheme 1. Synthesis of C_{60} derivatives with phenylazide (**5**) and phenylaziridine (**9**) group.

is readily prepared by the method of Prato and co-workers[22] and Wilson and co-workers.[23,24] This route provides a convenient approach to C_{60} derivatives with a secondary amine as an ideal site for the incorporation of further functionalization.

The synthesis of phenylazide derivative of fullerene was achieved as shown in Scheme 1. Dimethyl fulleropyrrolidine **2** (*cis*- and *trans*-mixture) was prepared by 1,3-dipolar cycloaddition[25] and then acylated with acid chloride **4** to give *cis*- and *trans*-C_{60}-phenylazide derivatives **5**, which can be easily separated by silica gel column chromatography.[26]

To synthesize the C_{60}-phenyldiazirine derivative **9**, we first attempted the reaction of fulleropyrrolidine **2** with an acid chloride, but this reaction did not give useful amounts of the desired product. Despite attempts to activate the acyl moiety by a succinimide group using 4-(3-trifluoromethylazirino)benzoic succinimide, product formation was not observed. In sharp contrast, however, the use of Yamaguchi reagent **8** to couple **6** and **2** gave good yields of *cis*- and *trans*-C_{60}-phenyldiazirine derivatives **9**.[27] These stereoisomers are readily separated by silica gel chromatography. Compounds **5** and **9** were characterized by spectroscopic methods.[28] The *cis*- and *trans*-stereochemistry of each compounds were determined according to the reported studies.[23,24]

In addition to the potential utility of fullerene-derived photoaffinity labels for elucidating the active site of C_{60} binding to enzymes such as GST and HIV-1 protease, the ability to selectively tag a protein or enzyme with fullerene may offer a new approach to the detection of biological molecules with high sensitivity. For example, an acidic isozyme of GST is specified as cancer expressing marker in liver cancers.[29,30] The ability to selectively tag such diagnostic enzymes with C_{60}, which has unique and useful chemical and photophysical properties, may offer a novel and rapid detection method for identifying trace amounts of enzyme present in a biological sample. These and other applications of the reported photoaffinity labeling reagents currently in progress.

In conclusion, we have described a concise and flexible route to fullerene-derived photoaffinity labels with potential utility in enzyme tagging and the elucidation of the binding sites of protein to C_{60}.

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25. To a solution of C_{60} (36 mg, 0.05 mmol) and D,L-alanine (9.2 mg, 0.10 mmol) in 1,2-dichlorobenzene (10 mL), acetaldehyde (11 mg, 0.25 mmol) was added and the mixture stirred at 120 °C for 20 h. The reaction process was checked by HPLC [silica gel column, solvent: benzene–EtOAc (10:1)]. The reaction mixture was purified by silica gel column chromatography (hexane–benzene–EtOAc) to give brown solid **2** (9.0 mg, 0.011 mmol, $y = 22\%$) as a *cis*- and *trans*-mixture.
26. To a solution of 4-azidobenzoic acid **3** (1.84 g, 11 mmol) in CH_2Cl_2 (5 mL), $SOCl_2$ (4.0 mL, 6.5 g, 55 mmol) in CH_2Cl_2 (5 mL) was added under argon atmosphere. Subsequently, dry DMF (1.5 mL) was added dropwise under Ar. After stirring for 2 h under Ar, the generation of acid chloride **4** was checked by TLC [solvent: hexane–EtOAc (1:1)] and then reaction mixture was filtered and concentrated in vacuo. To a solution of dimethyl fulleropyrrolidine **2** (*cis*- and *trans*-mixture, 20 mg, 0.025 mmol) in benzene (10 mL), acid chloride **4** (100 mg, 0.55 mmol) and pyridine 1 mL were added and the mixture stirred at 50 °C for 1 h. The reaction process was checked by TLC [benzene–EtOAc (1:1)], and then small amount of Et_3N was added. The products (*cis*- and *trans*-isomers) were separated by silica gel column chromatography (hexane–benzene–EtOAc) to give *cis*-**5** (8.9 mg, 9.5 μmol, $y = 38\%$) and *trans*-**5** (13.3 mg, 14.2 μmol, $y = 57\%$).
27. To a solution of 4-(3-trifluoromethylazirino)benzoic acid **6**, (9.7 mg, 0.042 mmol) with Et_3N (10 μL) in toluene (2 mL), 2,4,6-trichlorobenzoyl chloride (10 μL) was added and stirred under Ar at room temperature for 1.5 h. The reaction process was monitored by TLC [hexane–EtOAc (1:1)]. Subsequently, dimethyl fulleropyrrolidine **2** (10 mg, 12.6 μmol), DMAP 7 mg in toluene (4 mL) was added and then stirred under Ar at room temperature in dark condition for 2 days. The reaction process was monitored by TLC [benzene–EtOAc (1:1)] and then reaction mixture was purified by silica gel column chromatography (hexane–benzene–CH_2Cl_2) to give *cis*-**9** (2.5 mg, 2.5 μmol, $y = 20\%$) and *trans*-**9** (5.6 mg, 5.6 μmol, $y = 44\%$).
28. Selected spectroscopic data for *cis*-**5**: ^{1}H NMR ($CDCl_3$, 300 MHz): 2.28 (d, $J = 6.9$, 6H), 6.14 (q, $J = 6.9$, 2H), 7.23 (d, $J = 8.7$, 2H), 7.81 (d, $J = 8.7$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 936 ($[M-1]^-$), 720. *trans*-**5**: 2.21(d, $J = 6.0$, 6H), 5.74 (q, $J = 6.6$, 2H), 7.22 (d, $J = 8.4$, 2H), 7.99 (d, $J = 8.4$, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz): 19.9 (CH), 65.3 (CH3), 119.5 (CH), 130.2 (CH), 133.3–154.6 (C_{60}), 173.1 (CO); MALDI-TOF-MS (negative, matrix: DTT): 936 ($[M-1]^-$), 720; FT-IR (KBr): 2122 (N_3), 1670 (CO), 1600, 1260, 1182, 842, 756, 527 cm^{-1}. *cis*-**9**: ^{1}H NMR ($CDCl_3$, 300 MHz): 2.27 (d, $J = 6.7$, 6H), 6.08 (q, $J = 6.7$, 2H), 7.40 (d, $J = 8.5$, 2H), 7.81 (d, $J = 8.5$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 1003 ($[M-1]^-$), 720. *trans*-**9**: 2.23 (d, $J = 6.9$, 6H), 5.17 (q, $J = 6.9$, 2H), 7.40 (d, $J = 8.3$, 2H), 7.81 (d, $J = 8.3$, 2H); MALDI-TOF-MS (negative, matrix: DTT): 1003 ($[M-1]^-$), 720.
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Proteomic Analysis of Putative Latex Allergens

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Key Words
Proteomics · Allergenomics · Latex allergy · Allergen · Two-dimensional gel electrophoresis · Immunoblotting · In-gel digestion · Mass spectrometry · Database search

Abstract
Background: Extensive analysis of allergenic proteins is generally time-consuming and labor-intensive. Accordingly, a rapid and easy procedure for allergen identification is required. As sequence information on proteins and genes is accumulated in databases, it is becoming easier to identify a candidate protein using proteomic strategies, i.e. two-dimensional gel electrophoresis, site-specific fragmentation, mass spectrometry and then database search. In this study, we evaluated the usefulness of a proteomic strategy for identifying putative allergens through its application to latex proteins. ***Methods:*** Latex proteins were separated with two-dimensional gel electrophoresis, and putative allergens were visualized by IgE immunoblotting using pooled serum from latex-sensitive patients. The IgE-interactive proteins were cut out from the negatively stained two-dimensional gel and subjected to in-gel digestion by trypsin. Then the resulting peptides were analyzed with mass spectrometry. Based on the mass spectrometric data we obtained, the allergen candidates were assigned by a database search. ***Results:*** Five previously reported allergens and five new allergen candidates were identified with the proteomic approach without isolating the individual proteins. Less than 1 mg of crude latex protein was sufficient for the entire protocol. Because plural proteins can be processed in parallel, analysis of about 50 IgE-interactive proteins was accomplished within 1 week. ***Conclusions:*** Analysis of putative allergens with proteomic strategies (allergenomics) is a promising avenue for rapid and exhaustive research. The high resolving power of two-dimensional gel electrophoresis is superior to conventional gel electrophoresis. Moreover, the notable sensitivity and speed of mass spectrometry have pronounced advantages over the N-terminal sequencing that has generally been used for protein identification.

Introduction

Latex allergy has become a serious problem in medical settings since the late 1980s [1]. Intensive research on allergenic proteins responsible for this immediate-type hypersensitivity has revealed 13 officially registered latex allergens to date. It is notable that many of the latex allergens have something to do with the defense mechanisms of the rubber tree [2–6]. Defense-related proteins of higher plants are relatively conserved in the course of evolu-

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tion and therefore share the partial sequences indispensable for their function. Such a conserved sequence is sometimes called a functional domain. For example, hevein is one of the major latex allergens and contains a chitin-binding domain that is indispensable for its antifungal activity. A homologous chitin-binding domain has already been found in several antifungal proteins from various plant species. Class I chitinase is a representative containing a hevein-like domain. Because of this shared domain, class I chitinase is expected to be allergenic for hevein-sensitized patients [4–6]. Cross-reactivity between class I chitinase and hevein was indeed confirmed by several research groups [7, 8]. However, hevein cannot explain all of the cross-reactions of latex-allergic people to a wide range of fruits and vegetables (latex-fruit syndrome). Other undiscovered minor cross-reactive latex allergens must play a role in the latex-fruit syndrome. We need a powerful methodology in order to investigate such minor but significant allergens as well as major but hidden allergenic proteins.

As sequence data of proteins and genes are accumulated in databases, it is becoming easy to analyze candidate proteins rapidly and comprehensively using an effective separation technique coupled with mass spectrometry [9, 10]. This new strategy, aiming at comprehensive analysis of total proteins (proteome) in a cell or tissue, is now referred to as 'proteomics'. For the application of proteomic strategies to allergenic proteins in particular, the term 'allergenomics' has been proposed. In conventional approaches, we needed to isolate each allergenic protein to identify and fully characterize it. This process is usually complex, time-consuming and labor-intensive. However, in allergenomics, complex protein mixtures are effectively resolved with two-dimensional gel electrophoresis or the equivalent, and IgE-interactive proteins are detected by subsequent immunoblotting using pooled patients' serum. We can easily identify putative allergens through their site-specific degradation and the subsequent mass spectrometric analysis of the fragmented peptides and database search. The overall procedure is expected to be quicker and less labor-intensive than any procedures used to date. Moreover, we can analyze numerous IgE-interactive proteins in parallel. Semiautomatic analysis of allergen candidates is also possible. From these features, allergenomics is expected to be a key technique for fast, effective and comprehensive analysis of IgE-interactive proteins.

A few examples of the application of proteomic strategies to pollen allergens have been published in recent years [11, 12]. However, the effectiveness of allergenomics has not been fully demonstrated yet. In this study, we evaluated the usefulness of allergenomics through the analysis of latex proteins. Latex allergens have been extensively studied using traditional biochemical methods [1]. Nevertheless, minor latex allergens that have been overlooked and new cross-reactive allergens pertinent to the latex-fruit syndrome remain to be scrutinized. The advantages of allergenomics in analyzing these troublesome allergens are demonstrated below.

Subjects and Methods

Subjects

Six latex-sensitive patients and four control volunteers provided their serum for this study. Latex-allergic patients (table 1) were diagnosed from their clinical history of allergy to natural rubber-containing products, positive results of a skin prick test (SPT) using crude extract of latex proteins [13] and scores from a latex-specific IgE radioallergosorbent test (CAP RAST, Pharmacia Diagnostics). When a subject with a convincing clinical history of latex allergy did not show positive reactions on the SPT, a gradual provocation test to a brand of highly allergenic latex gloves was carried out for definite diagnosis. Three of the control subjects were medical personnel who are exposed constantly to various kinds of products made from natural rubber. One control subject was a homemaker suffering from erythema nodosum but with no background of allergies. They were confirmed not to have a latex allergy from the diagnostic criteria mentioned above. Informed consent was obtained from all of the participants before collecting their blood.

Extraction of Latex Proteins

Latex proteins were extracted from nonammoniated latex (NAL) harvested from a rubber tree (*Hevea brasiliensis*, clone RRIM600) at the Rubber Research Institute of Malaysia. The NAL was immediately frozen at the site of collection and dispatched to Japan. By thawing and pressing the frozen latex, we obtained a brown liquid that oozed out from it. The liquid (about 100 ml) was supplemented with two tablets of protease inhibitor cocktail (Complete Mini, Roche Applied Science) and dialyzed against running water using Spectra/Por 3 membrane tubing (cutoff 3.5 kD; Spectrum Laboratories, Inc.) overnight. The dialyzate was then lyophilized and reconstituted in phosphate-buffered saline (pH 7.4). After condensation of the solution using Centriprep-10 (cutoff 10 kD; Millipore Co.), the concentrate was ultracentrifuged at 40,000 rpm for 24 h at 4°C (P70AT2 rotor and CP 65β ultracentrifuge, Hitachi Koki Co., Ltd.). The protein content of the supernatant was determined with a BCA Protein Assay Kit (Pierce Chemical Co.) as 13.9 mg/ml. This supernatant was submitted to later study.

Two-Dimensional Gel Electrophoresis

Latex proteins (250 μg) extracted from NAL were diluted in 250 μl of Destreak Rehydration Solution (Amersham Biosciences) supplemented with IPG buffer (0.1%, pH 3–10, nonlinear; Amersham). This solution was used for overnight rehydration of an immobilized pH gradient gel strip (Immobiline DryStrip, Amersham; pH 3–10, nonlinear, 13 cm). Then the strip was submitted to isoelectric focusing on a Multiphor II Electrophoresis Unit (Amersham) at

Table 1. Summary of latex-allergic patients who provided their blood for IgE immunoblotting

Patient No.	Age years	Gender	Occupation	Total IgE IU/ml[1]	Latex-specific IgE IU/ml (score)[2]	Latex SPT	Positive SPT	Other allergic symptoms
1	26	F	nurse	49.1	14.0 (3)	4+	Japanese horseradish	OAS to Japanese horseradish
2	34	F	housewife	1,110	4.50 (3)	4+	avocado, grapefruit	atopic dermatitis, bronchial asthma
3	22	F	dental hygienist	273	2.23 (2)	3+	tomato, spices	OAS, atopic dermatitis, pollinosis
4	25	M	medical doctor	15,500	82.0 (5)	3+	avocado	atopic dermatitis, atopic rhinitis
5	25	F	nurse	88.7	2.81 (2)	3+	–	OAS to melon
6	28	F	nurse	206	15.0 (3)	4+	–	OAS, atopic dermatitis, atopic rhinitis

OAS = Oral allergy syndrome.
[1] IgE radioimmunosorbent test (Pharmacia).
[2] CAP radioallergosorbent test (Pharmacia).

20 °C. Proteins were focused by applying programmed voltage as follows: 0–300 V in 1 min, 300–3,500 V in 1.5 h and 3,500 V for 7 h. Thereafter, the gel strip was submitted to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as the second dimension of electrophoresis. Before applying to SDS-PAGE, the gel strip was incubated at room temperature in 7.5 ml of equilibration buffer (Amersham) supplemented by dithiothreitol (1%) for 30 min, then in the same equilibration buffer containing iodoacetamide (1%) instead of dithiothreitol for 30 min. The gel strip was placed on top of a slab gel (10% acrylamide, 14 × 14 cm) and fixed with agarose (0.5%). The second electrophoresis was carried out by applying a constant current of 30 mA for 30 min then 70 mA for 5 h at 20 °C using an SE 600 Ruby Electrophoresis Unit (Amersham).

Immunoblotting

Two-dimensionally separated proteins were transferred onto a polyvinylidene fluoride membrane (14 × 14 cm; Immobilon-P, Millipore) using a semidry transfer cassette (ATTO Co.) by applying a constant current of 200 mA for 1.5 h. SDS (0.1%) was added to each of the discontinuous blotting buffers containing methanol (10%) for efficient transfer of proteins with high molecular weight or a basic isoelectric point. After washing with Tris-buffered saline (TBS; pH 7.4) supplemented with Tween-20 (TBS-T; 0.1%), the membrane was soaked in a chilled and degassed 10 m*M* $NaIO_4$-50 m*M* acetate buffer (pH 4.7) and incubated at 4 °C in the dark overnight. Then the membrane was repeatedly washed with TBS-T and incubated at room temperature in a blocking buffer (Block Ace, Dai-Nippon Pharmaceutical Co.) for 5 h. Following washing with TBS-T, the membrane was incubated overnight in the pooled patients' serum (20 ml) diluted 1/20 in TBS-T at room temperature. In the control experiment, the membrane was incubated in the pooled control serum (20 ml) diluted 1/20 in TBS-T. The next day, the membrane was washed again with TBS-T and incubated at room temperature in a solution of peroxidase-labeled affinity-purified antibodies to human IgE(ε) (Kirkegaard & Perry Laboratories, Inc.) diluted 1/10,000 in TBS-T for 1 h. After repeated washing with TBS-T, IgE-recognized protein spots were visualized using an ECL Western Blotting Detection System and Hyperfilm ECL (Amersham).

In-Gel Digestion

Two-dimensionally separated proteins on the gel were visualized by negative staining [14] using a Zinc Stain Kit (Bio-Rad Laboratories). Protein spots that were judged as IgE-interactive from the immunoblotting with pooled patients' serum were cut out from the gel (highlighted spots in fig. 1A). After treatment with Zinc Destain Solution (Bio-Rad) as instructed by the manufacturer's protocol, the protein spots were submitted to tryptic digestion using a Montage In-Gel Digest96 Kit (Millipore). Digestion proceeded at 30 °C overnight following the manufacturer's recommendations except that *n*-octyl-β-*D*-glucoside (Dojindo Laboratories) was added to the digestion mixture (final 0.1%) for efficient recovery of the fragmented peptides [15].

Mass Spectrometry and Database Search

Fragmented peptides were desalted by means of a reversed-phase microcolumn (ZipTip μC_{18}, Millipore) as instructed by the manufacturer, and eluted from the column with the help of α-cyano-4-hydroxy cinnamic acid solution on a sample plate for mass spectrometry. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) was performed on a 4700 Proteomics Analyzer (Applied Biosystems Inc.). Positive-ion mass spectra of tryptic peptides were recorded on the reflectron mode of the instrument. The information from these spectra (peptide mass maps, fig. 2A) was used for a database search of the original proteins with the peptide mass fingerprinting (PMF) mode of a search engine (Mascot, Matrix Science). Further, tandem mass spectra (MS/MS spectra) of prominent peptide ions on the peptide mass map were recorded on the time-of-flight (TOF)-TOF mode of the instrument [16]. The information (peptide sequence tag; PST) from such tandem mass spectra (fig. 2B) was employed for a database search with the MS/MS Ion Search mode of Mascot for more precise identification of IgE-interactive proteins. National Center for Biotechnology Information (nonredundant) (NCBInr) was selected as the first database to be searched using Mascot. When there was no candidate with enough certainty, SwissProt and Expressed Sequence Tags (EST) *(H. brasiliensis)* were employed as the second and third databases to be searched, respectively. The taxonomy of the database category was set to *Viridiplantae* (Green Plants), and carbamidomethyl cysteine was selected as a fixed modification of fragmented peptides. The number of allowable missed cleavages in tryptic digestion was set at

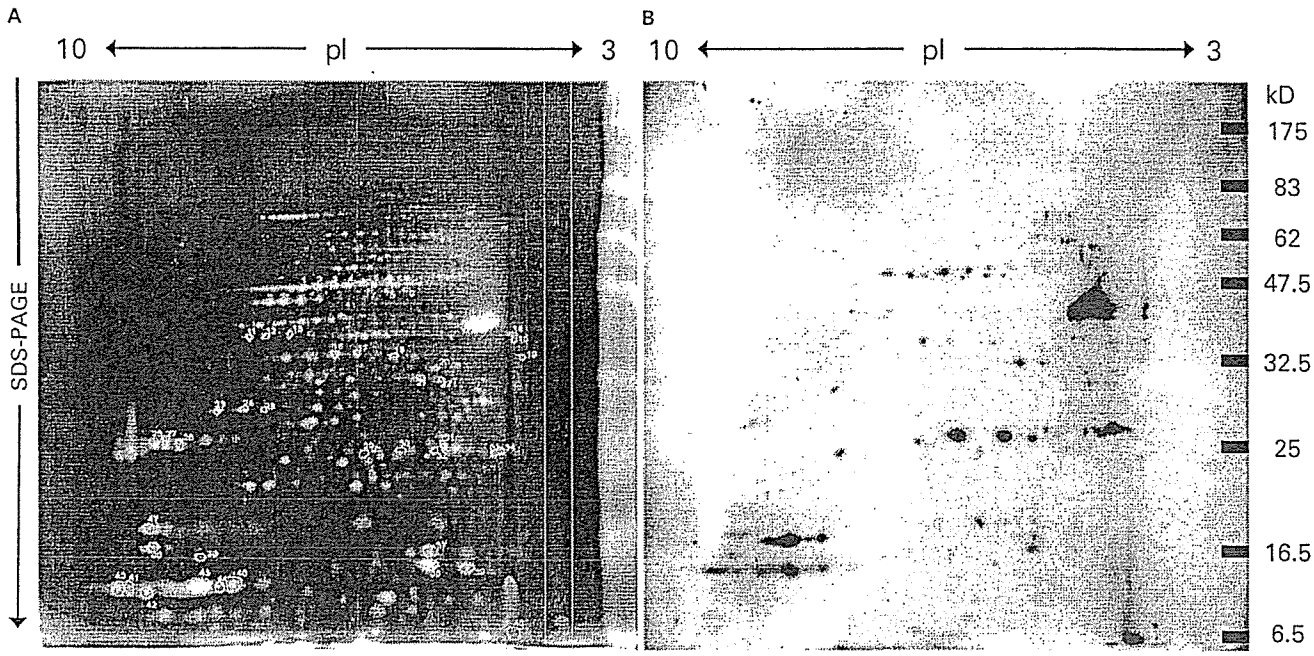

Fig. 1. Latex protein (**A**) and IgE-interactive protein (**B**) profiles on two-dimensional gels. Latex proteins extracted from NAL were first separated according to their isoelectric point (pI) on an immobilized pH gradient gel strip (pH 3–10, nonlinear). Second, the partially separated proteins were further resolved according to their molecular weight on a 10% acrylamide slab gel (SDS-PAGE). Two-dimensionally separated proteins were visualized by negative staining (**A**). IgE-interactive proteins submitted for subsequent in-gel digestion are highlighted and numbered (No. 1–47). For IgE immunoblotting (**B**), two-dimensionally separated proteins were transferred onto a membrane and treated by $NaIO_4$ for degradation of the possible carbohydrate structures. After blocking the unoccupied sites, the membrane was incubated in the pooled serum of 6 latex-sensitive patients. Spots of proteins that interacted with the IgE antibodies were then visualized by reaction with peroxidase-labeled anti-human IgE antibodies. The enzyme activity was recorded on a film using a chemiluminescent detection system. The migrating position and molecular weight of each marker protein are shown on the right side of the gel.

1. Mass error tolerance was set to plus or minus 0.5 D for PMF. In the MS/MS Ion Search mode, the mass error tolerance of a precursor ion was set to plus or minus 1.0 D, and plus or minus 0.5 D for the product ions. All the peptide masses mentioned in this article are monoisotopic masses. Sequentially homologous proteins from other organisms were searched using the Basic Local Alignment Search Tool (BLAST) search engine-based overall sequence of the top candidate picked up from the EST *(H. brasiliensis)* database.

Results

Two-Dimensional Immunoblotting of Latex Proteins

Proteins extracted from NAL were separated with two-dimensional gel electrophoresis. Its high resolution was visualized by negative staining of the gel (fig. 1A). More than 300 distinct protein spots, each with a different isoelectric point or molecular weight, were detected. This resolving power is far superior to the conventional SDS-PAGE or isoelectric focusing where proteins are separated one-dimensionally. IgE-interactive proteins were selectively detected by reaction with the pooled patients' serum following $NaIO_4$ treatment of a membrane binding the two-dimensionally separated proteins. $NaIO_4$ oxidatively degrades the carbohydrate structures of glycoproteins that tend to result in false-positive reactions of IgE antibodies by acting as monovalent antigens [17, 18]. Monovalent antigens cannot form a bridge-like structure of IgE antibodies attached to the specific receptor on a sensitized cell, and therefore they are believed not to provoke any allergic responses. We clearly detected more than 50 spots of proteins interacting with the IgE antibodies owing to the high resolving power of two-dimensional gel electrophoresis and the advanced sensitivity of the chemiluminescent detection system compared to conventional color-developing systems (fig. 1B). No significant spots of IgE-interactive proteins were detected when the pooled control serum was employed for immunoblotting (data not shown).

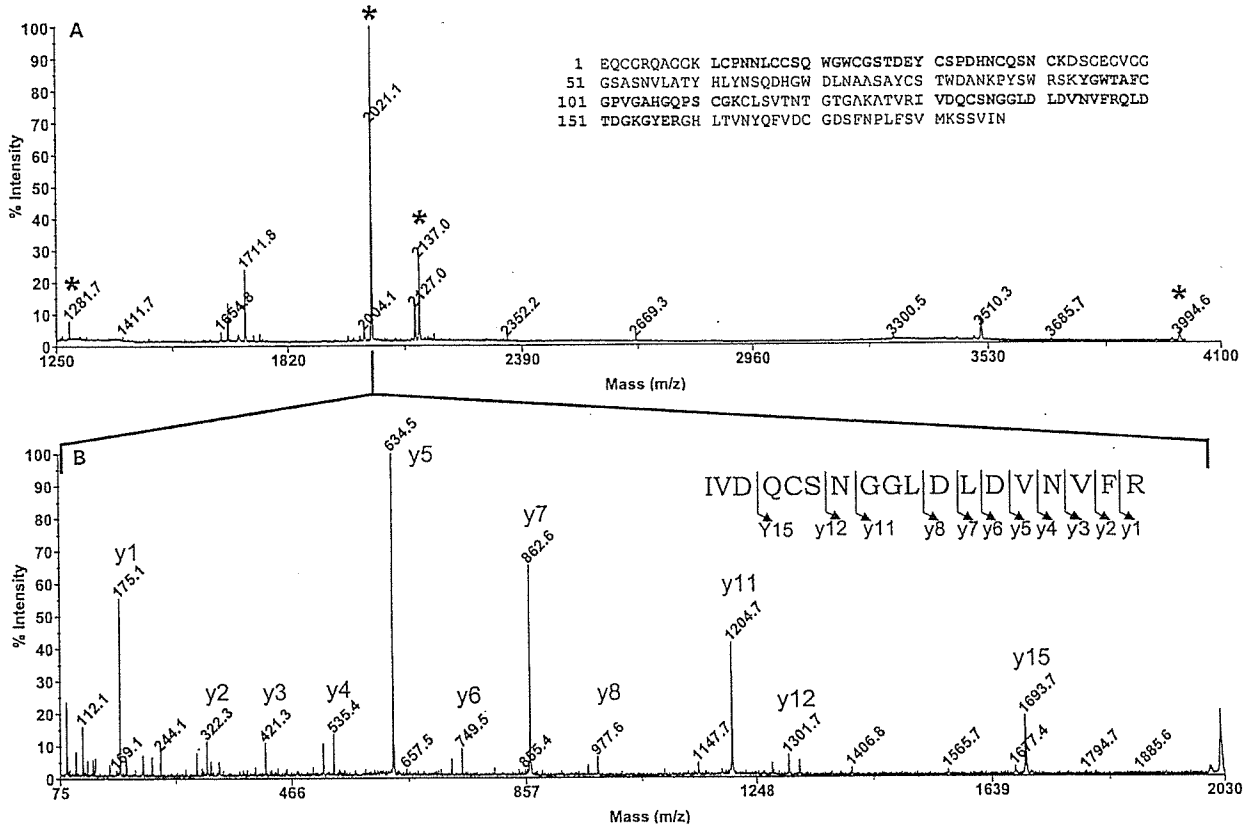

Fig. 2. Positive-ion reflectron MALDI-TOFMS spectrum (**A**) of tryptic peptides derived from an IgE-interactive protein (spot No. 38 in fig. 1A) and an MS/MS spectrum (**B**) of a prominently detected peptide ion (m/z 2021.1) on the peptide mass map. The spot of a putative allergen (No. 38) was cut out from a negatively stained two-dimensional gel (fig. 1A) and site-specifically cleaved by trypsin. The fragmented peptides were desalted using a reversed-phase microcolumn and spotted on a sample plate. α-Cyano-4-hydroxy cinnamic acid was used as a matrix for ionization. A peptide mass map (**A**) was used for PMF. This spectrum is a result of accumulation of 2,000 laser shots. The peptide ions assigned in the PMF search are labeled by asterisks, and their position is shown in boldface in the entire sequence of the top candidate (insert). The MS/MS spectrum (**B**) was recorded on the TOF-TOF mode of the instrument (4700 Proteomics Analyzer) and used for reading the PST. This MS/MS spectrum shows the accumulation of 5,000 laser shots. A partial sequence read from this spectrum is depicted as an insert with the name of the fragment ions (y series) detected. Based on a series of mass spectra including the ones shown in this figure, the original antigen (spot No. 38 in fig. 1A) was identified as a prohevein-related protein by database search (table 2).

In-Gel Digestion of Putative Allergens and Mass Spectrometry of Tryptic Peptides

Forty-seven spots of latex proteins that interacted with IgE antibodies from patients' sera were cut out from a two-dimensional gel (fig. 1A). With the help of the microtiter plate-based format of an in-gel digestion kit (Montage In-Gel Digest96 Kit), the putative allergens in the gel pieces were simultaneously digested by trypsin with less risk of keratin contamination. By adding *n*-octyl-β-*D*-glucoside to the digestion buffer, we could significantly improve the quality of mass spectra of fragmented peptides (fig. 2A). Desaltation of tryptic peptides using a reversed-phase microcolumn (ZipTip μC_{18}) was also helpful for improving the signal-to-noise ratio of the mass spectra (fig. 2A). We used a MALDI-TOF-TOF instrument for mass spectrometric analysis of tryptic peptides. Though the quality of MS/MS spectra is generally inferior to the spectra obtained on an electrospray ionization-based tandem mass spectrometer, the operation is less labor-intensive and very quick. The sensitivity of the mass spectrome-

Table 2. Summary of IgE-interactive proteins identified by allergenomics with a Mascot search

Spot No.	Name of the top candidate	Method used	Database used	Total score	Sequence coverage, %	Accession No.	Calculated Mr, kD	Calculated pI	Observed Mr, kD	Observed pI
1	UDP-glucose pyrophosphorylase	PST	NCBInr	46	2	gi|12585472	51.6	5.92	49.0	6.6
3	UDP-glucose pyrophosphorylase	PST	NCBInr	24	2	gi|12585472	51.6	5.92	49.0	6.4
4	UDP-glucose pyrophosphorylase	PST	NCBInr	58	6	gi|8099155	50.9	6.10	49.0	6.3
5	enolase 2 (Hev b 9)	PST	NCBInr	32	5	gi|14423687	48.1	5.92	49.0	6.3
7	enolase 1 (Hev b 9)	PMF	NCBInr	52	22	gi|14423688	48.0	5.57	47.5	6.0
	enolase 1 (Hev b 9)	PST	NCBInr	38	5	gi|14423688	48.0	5.57		
10	patatin-like latex allergen 2 (Hev b 7)	PMF	NCBInr	156	37	gi|7442023	43.0	5.00	40.0–43.5	4.6–5.5
	patatin-like latex allergen 1 (Hev b 7)	PST	NCBInr	68	13	gi|7442022	43.1	5.12		
21	isoflavone reductase[1]	PST	EST(latex)	201	15	gi|29054556	27.0	5.32	32.5	5.6
22	isoflavone reductase[1]	PST	EST(latex)	69	15	gi|29054556	27.0	5.32	32.5	5.4
26	hevamine A	PMF	NCBInr	151	54	gi|1311006	29.9	8.44	29.0	9.4
	hevamine A	PST	NCBInr	156	19	gi|1311006	29.9	8.44		
27	hevamine A	PMF	NCBInr	151	65	gi|1311006	29.9	8.44	29.0	9.3
	hevamine A	PST	NCBInr	240	32	gi|1311006	29.9	8.44		
28	hevamine A	PMF	NCBInr	137	54	gi|1311006	29.9	8.44	29.0	9.1
	hevamine A	PST	NCBInr	113	13	gi|1311006	29.9	8.44		
33	class I chitinase[1] (Hev b 11-related)	PST	EST(latex)	87	7	gi|29054149	23.5	8.28	26.5	4.2
34	class I chitinase[1] (Hev b 11-related)	PST	EST(latex)	121	15	gi|29054149	23.5	8.28	26.5	4.0
36	hevein precursor (Hev b 6-related)	PST	NCBInr	36	8	gi|123062	22.7	5.63	16.5	8.6
37	prohevein (Hev b 6)	PST	NCBInr	69	20	gi|2832430	20.9	5.64	17.0	5.6
38	prohevein (Hev b 6)	PMF	NCBInr	62	43	gi|2832430	20.9	5.64	15.0	5.6
	prohevein (Hev b 6)	PST	NCBInr	198	20	gi|2832430	20.9	5.64		
39	prohevein (Hev b 6)	PST	NCBInr	127	20	gi|2832430	20.9	5.64	15.0	5.3
40	rotamase	PST	SwissProt	21	6	Q39613	18.5	8.36	11.0	9.8
41	rotamase	PMF	SwissProt	38	27	Q39613	18.5	8.36	11.0	9.6
	rotamase	PST	NCBInr	50	6	gi|3334157	18.5	8.36		
42	rotamase	PST	NCBInr	98	8	gi|118104	18.6	8.91	10.5	9.3
43	prohevein (Hev b 6)	PMF	NCBInr	44	32	gi|2832430	20.9	5.64	11.0	8.7
	prohevein (Hev b 6)	PST	NCBInr	201	26	gi|2832430	20.9	5.64		
44	pseudo-hevein (Hev b 6-related)	PMF	NCBInr	49	33	gi|6562381	21.0	7.98	11.0	8.4
	prohevein (Hev b 6)	PST	NCBInr	114	20	gi|2832430	20.9	5.64		
45	pseudo-hevein (Hev b 6-related)	PMF	NCBInr	39	33	gi|6562381	21.0	7.98	11.0	8.0
	prohevein (Hev b 6)	PST	NCBInr	196	20	gi|2832430	20.9	5.64		
46	thioredoxin h	PST	NCBInr	48	13	gi|14485509	13.2	4.83	6.5	3.9
47	citrate-binding protein precursor	PST	NCBInr	72	10	gi|32363139	27.5	9.09	17.0	9.4

Mr = Molecular weight; pI = isoelectric point.
[1] Sequentially homologous protein was searched using BLAST.

try is also notable. We could easily obtain positive-ion peptide mass maps (fig. 2A) and MS/MS spectra of prominent ions (fig. 2B) from as small as several hundred femtomoles of tryptic peptides on a sample plate.

Identification of Putative Allergens by Database Search

Based on a peptide mass map of the tryptic peptides and MS/MS spectra of the intense peptide ions, the proteins in question were identified by database search. The identified putative allergens are listed in table 2, with the total score and sequence coverage rate showing the certainty of the protein candidate. Thanks to the high resolution of two-dimensional gel electrophoresis and the remarkable sensitivity of MALDI-TOFMS, we could identify five new allergen candidates (UDP-glucose pyrophosphorylase, isoflavone reductase, rotamase, thioredoxin and citrate-binding protein) and five previously reported latex allergens or their relatives (Hev b 9, Hev b 7, Hev b 11, Hev b 6 and hevamine) without isolating each protein

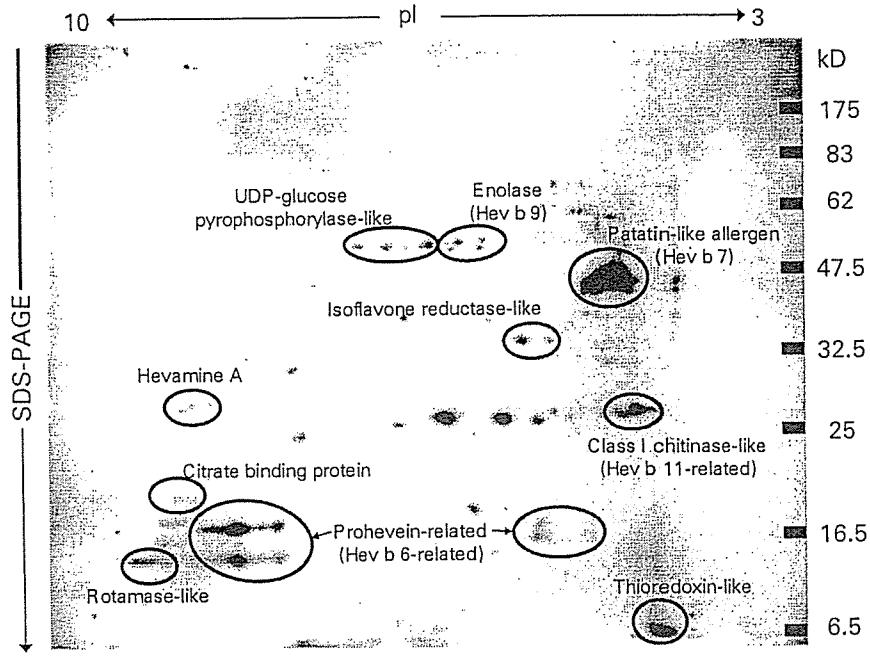

Fig. 3. Putative latex allergens identified by a proteomic strategy. The names of the top candidates are shown on an IgE immunoblot (same as in fig. 1B). Spots enclosed by an ellipse are IgE-interactive proteins assigned to the same or closely related entries (isoallergens) by database search. More information on each antigenic protein is presented in table 2.

(table 2, fig. 3). A few allergens were identified by database search with the PMF mode of Mascot, but the searching based on PST was much more powerful for protein identification. From the MS/MS spectra of tryptic peptides derived from a protein, we could obtain information on the partial sequences (fig. 2B) that would be valuable for designing the oligonucleotide probes as well as for the database search. The BLAST search was also effective in finding a protein candidate that is sequentially comparable to a DNA fragment registered in the EST database *(H. brasiliensis)*. The entire protocol, from two-dimensional gel electrophoresis to the database search, was accomplished quickly. We were able to analyze about 50 IgE-interactive latex proteins within 1 week.

Discussion

In this study, we evaluated the usefulness of a proteomic strategy for analyzing putative allergens (allergenomics) through application to latex proteins. Latex proteins were first separated two-dimensionally according to their isoelectric point and molecular weight under denaturing conditions. Two-dimensional gel electrophoresis appeared to be remarkably superior to SDS-PAGE with regard to its high resolution. In conventional SDS-PAGE, proteins were separated based solely on their molecular weight under a denaturing condition. Therefore, a seemingly single band on an SDS-PAGE gel frequently contains several protein entities. This poor resolution might bring about erroneous identification of IgE-interactive proteins. Additionally, faint structural differences of isoallergens are hardly reflected on SDS-PAGE gel and subsequent IgE immunoblot. In contrast, we can detect slight structural differences of isoallergens as distinct spots on a two-dimensional gel and subsequent IgE immunoblot. Hev b 7 isoallergens are one such example (fig. 3, table 2, spot No. 10). Historically, the reproducibility of two-dimensional gel electrophoresis has been a critical problem in applying this technique to research of complex protein mixtures. However, the appearance of commercially available immobilized pH gradient gels has solved a large part of this difficulty. The effectiveness of two-dimensional gel electrophoresis for analysis of allergenic proteins has already been demonstrated in previous studies [19–23]. Nevertheless, this technique has not attracted the attention of many researchers, due to the lack of a convenient identification method for the resolved proteins. N-terminal sequence analysis of a purified protein has been the first choice for identification [10, 20], but this technique is time-consuming and requires a relatively large quantity of purified sample. Putative allergens avail-

able only in minute amounts were therefore not readily amenable to this approach.

As the sequence data of proteins and genes accumulate in databases, the situation is changing. Candidate proteins are becoming easier to identify after site-specific fragmentation and mass spectrometric analysis of the component peptides [9, 10]. Mass spectrometry is so sensitive that we can analyze putative allergens on quantities as small as the nanogram or several hundred-femtomole level. Intensive research of such a small quantity of proteins was impossible with traditional Edman degradation-based N-terminal sequencing. The speed of mass spectrometric analysis is also advantageous. We can obtain thousands of mass spectra and MS/MS spectra within a day using a MALDI-TOFMS instrument. Semiautomated analysis of fragmented peptides from spotting on a sample plate to output of the mass spectra is also possible. By combining with high-resolution two-dimensional gel electrophoresis, the mass spectrometric approach will certainly become the most powerful means for fast and exhaustive analysis of IgE-interactive proteins [24].

The efficiency of allergenomics was substantiated in this study through the analysis of latex proteins. We were able to identify five new allergen candidates as well as five previously reported allergens without any difficulties (fig. 3, table 2). The required starting material was less than 1 mg. In addition, the entire analysis was completed within 1 week; two-dimensional gel electrophoresis and subsequent IgE immunoblotting usually takes not more than 5 days. Referring to the immunoblotting result with pooled patients' serum, IgE-interactive proteins were cut out from a two-dimensional gel and digested by trypsin overnight. A more rapid digestion protocol for proteins is also applicable [25]. The proteins in question were identified the next day from mass spectrometric analysis of the resulting fragments and subsequent database search. These analytical steps for each IgE-interactive protein proceed in parallel with the help of a microtiter plate-based tryptic digestion regime. In this way, allergenomics was concretely verified as a quick and effective strategy for exhaustive analysis of IgE-interactive proteins from various species.

We should point out at this stage that IgE-interactive proteins or antigens identified with allergenomics are just putative allergens or allergen candidates. IgE-binding activity of a protein is one of the prerequisites for it to be an allergen and does not necessary indicate its actual allergenicity. Some IgE-interactive proteins are indeed asymptomatic but others are symptomatic [17, 18]. Therefore, the actual allergenicity of putative allergens must finally be confirmed with other techniques like SPT or histamine release test. This situation is common to almost all the allergen identification procedures reported to date where allergen candidates are screened based on their IgE-binding activity.

Even though allergenomics is very promising, as already illustrated, some of the latex antigens were not identified by database search, as shown in figure 3, despite their clear detection in IgE immunoblotting. There are two possible reasons for this failure. One possibility is that the quantity of IgE-interactive protein was too small to gain meaningful data regardless of the high sensitivity of the mass spectrometry. Another possibility is that data on the sequence of the antigen in question had not been registered in the databases used. The genome of the rubber tree *(H. brasiliensis)* has not been sequenced thoroughly. Therefore, we cannot identify some of the latex proteins by the proteomic strategy in principle. If we analyze proteins from rice or *Arabidopsis,* this is not the case because the genome projects of these species have already been finished. Even when the sequence of an IgE-interactive protein is not included in databases, we can search sequentially homologous proteins from other species using the partial sequences of tryptic peptides or cDNA fragments. Moreover, the gene coding the putative allergen can be fully sequenced by cloning the corresponding cDNA using oligonucleotide probes designed from the revealed partial sequences. These prospects for further research also suggest the versatility of allergenomics.

Acknowledgement

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CHANGE OF THE CELLULAR FUNCTION BY CONNEXIN GENE TRANSFECTION IN A HEPATOMA CELL LINE

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Abstract

Connexin 32 (Cx32) is the main gap junction protein in hepatocytes and plays an important role in the regulation of liver gap junctional communication (GJIC). In this study, the human Cx32 gene was transfected in a hepatoma cell line (HepG2) that is aberrant expression of Cx32 and deficient in GJIC. Cx32-transfected HepG2 showed the increased GJIC comparing with HepG2 and the vector-transfected HepG2. Furthermore, the liver functions of ammonia removal activity of HepG2 were remarkably enhanced with Cx32 gene transfection. It may be expected to improve the cellular functions of the hepatoma cell line by Cx32 gene transfection and serve to develop an efficacious bioartificial liver.

Introduction

A cell-based biohybrid artificial liver (BAL) is a promising approach to support patients with acute liver failure[1]. To overcome worldwide shortage of donor organs and avoid xenozoonosis risk, a hepatoma cell line HepG2 derived form the human-origin cell has growth characteristic and are less severe antigenecity, and then has already been used for developing the BAL[2]. Although HepG2 keeps liver-specific functions well among hepatoma cell lines, the activities of the liver-specific functions in HepG2 were far lower comparing with these of primary hepatocytes[3]. On the other hand, gap junction intercellular communication (GJIC) is considered to play an essential role in the control of proliferation, differentiation and homeostasis of various cells. In the liver, hepatocytes are coupled to each others by gap junctions and GJIC is necessary for liver homeostasis growth control and signal transfer, especially related to glycogen

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