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THE HUMAN ERYTHROCYTE MEMBRANE CONTAINS A NOVEL 12-kDa INOSITOLPHOSPHATE-BINDING PROTEIN THAT IS AN IMMUNOPHILIN

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A 12-kDa inositolphosphate-binding protein has been identified as a component of the human erythrocyte membrane. Its robust peptidylprolyl *cis-trans* isomerase activity that is strongly inhibited by the immunosuppressant drugs FK506 and rapamycin indicates that it is an immunophilin belonging to the FKBP class. The finding that its peptidylprolyl *cis-trans* isomerase activity is also strongly inhibited by nanomolar concentrations of the second messengers inositol 1,4,5-trisphosphate (IP_3) and inositol 1,3,4,5-tetrakisphosphate (IP_4) suggests that IP_3 and IP_4 could be physiological ligands for this membrane-associated immunophilin.

IP_3 and IP_4 are widely distributed second messengers that participate in signal transduction processes involving Ca^{2+} oscillations (1-3). Although the human erythrocyte membrane contains enzymes that metabolize IP_3 (4-7), a role for the inositolphosphates in this cell has not been identified. We have, therefore, examined solubilized preparations of human erythrocyte membranes for proteins that bind one, or both, of the second messengers. These studies resulted in the identification of a novel 12 kDa inositolphosphate-binding protein. Finding that it was associated with a protein kinase activity that appeared to catalyze its phosphorylation suggested a possible homology with the 12 kDa immunophilin, FKBP12, which has been shown to associate with specific phosphoryl-transfer enzymes including protein kinases. This communication describes some of our findings concerning this 12 kDa membrane-associated inositolphosphate-binding protein that has the characteristics of an immunophilin of the FKBP class.

Materials and Methods

The peptide substrate used in the peptidylprolyl *cis-trans* isomerase determinations, Suc-Ala-Leu-Pro-Phe-pNA, was obtained from Bachem Feinchemikalien AG, Bubendorf, Switzerland. Anti-phosphotyrosine (clone PT-66) and anti-phosphoserine (clone PSR-45) were obtained from Sigma Chemical Company, St. Louis, Mo.

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Membrane Isolation, Solubilization, and Gel Filtration

Human erythrocytes were obtained from normal volunteers in accordance with protocols and procedures approved by our Internal Review Board. Erythrocyte membranes were isolated by hypotonic lysis and subsequently solubilized using 1.0% Nonidet P-40, 30 min, 4°. The resulting supernatant was centrifuged (42,000 x g, 4°) for 120 min and 6.5 ml aliquots of the resulting supernatant (adjusted to 1.0-1.2 mg protein/ml) were applied to a 1.5 x 90 cm column of Sephacryl S-300 that was eluted using 50 mM HEPES buffer (pH 7.5) containing 1.0 mM magnesium acetate, 5 mM 2-mercaptoethanol and 0.1% Nonidet P-40. Routinely, 110 fractions, 1.8 ml each, were collected although most of the protein is obtained in the first 65-70 fractions.

Photoaffinity Labeling Using an Inositolphosphate Analogue

The photoaffinity-labeling probe had been synthesized by coupling (±)-(1R,3R,4R)-*trans*-N-(2-aminoethyl)-3,4- bis(phosphonyloxy)cyclohexane-1-carboxamide and succimido 4-azidosalicylate, as reported by Jina et al. (8). The arylazido ring was subsequently labeled with ^{125}I using the solid state reagent IODO-BEADS with $Na^{125}I$ (0.47 nmol, 1.0 mCi), according to the authors' (8) suggestions. Concentrated (6-fold) Sephacryl S-300 column fractions to be examined were first dialyzed against 50 mM HEPES buffer (pH 7.4) containing 0.1% Nonidet P-40 to remove the 2-mercaptoethanol. Aliquots (130 μl) were then pre-incubated in the dark with the labeled probe (approximately 2 x 10^7 dpm) for 60 min, at 4°, followed by irradiation for 2 min with 254 nm light (Mineralight UVSL-25) at a distance of 3.5 cm. The unused probe was destroyed by the addition of 2-mercaptoethanol (final concentration, 10 mM) in 100 mM Tris-HCl buffer (pH 6.7) containing 0.1% Nonidet P-40. Reaction mixtures were applied to 3-17% gradient, non-denaturing (Nonidet P-40) polyacrylamide gels in order to remove non-covalently bound ^{125}I and partially resolve the labeled proteins. Autoradiograms prepared from the non-denaturing gels indicated the locations of covalently labeled proteins. These gels were sectioned to isolate the labeled components and the radiolabeled gel segments were inserted at the top of 10-20% gradient SDS polyacrylamide gels. Autoradiograms of the SDS gels identified proteins that had bound the labeled inositolphosphate analogue. This procedure was used in examining combined, concentrated Sephacryl S-300 column fractions #35-#40, #41-#44, #45-#50, #51-#56, aliquots of the total Nonidet P-40 supernatant, and aliquots of the particulate from this solubilization. Histones and BSA were routinely used as negative controls.

Determination of Protein Kinase Activity

Protein phosphorylation reactions were conducted in 50 mM HEPES buffer (pH 7.4), 5.0 mM 2-mercaptoethanol, and 0.1% Nonidet P-40. Reaction mixtures contained 5.0 μM [γ-^{32}P]ATP (6.0-7.0 x 10^6 dpm) and 5.0 mM Mg^{2+} when examing 120 μl aliquots of Sephacryl S-300 column fractions or ATP as indicated and 10 mM Mg^{2+} when examining 90 μl aliquots of a suspension of immunoprecipitated, immobilized proteins. After 30 min at 30°, the phosphorylation reaction was stopped by the addition of SDS (final concentration, 1%) in 50 mM Tris-HCl buffer (pH 6.8) containing 2 mM 2-mercaptoethanol. Samples were then incubated in a boiling water bath for 10 min and applied to SDS polyacrylamide gels.

When proteins were to undergo limited phosphorylation prior to their immunoprecipitation with anti-phosphotyrosine, 1250 μl of combined, concentrated Sephacryl S-300 column fractions were incubated with 2 μM unlabeled ATP and 10 mM Mg^{2+} in the presence of 50 mM HEPES buffer (pH 7.4) containing 5.0 mM 2-mercaptoethanol and 0.1% Nonidet P-40. Total volume was 1400 μl. The reaction was stopped after 15 min at 30° by the addition of EDTA (final concentration, 16 mM).

Immunoprecipitation with a Monoclonal Antibody against Anti-phosphotyrosine

The (1400 μl) preparation that had undergone limited phosphorylation (described above) was incubated for 16 h at 4°, with shaking, with 500 μl of a suspension of agarose-linked anti-phosphotyrosine. The reaction medium was then centrifuged and the immunoprecipitated, immobilized proteins were washed 5 times with 1.0 ml aliquots of 50 mM HEPES buffer (pH 7.4) containing 50 mM sodium chloride, 20% glycerol, and 0.1% Nonidet P-40. Following the final centrifugation, the immobilized proteins were suspended in approximately 2.5 ml of the appropriate buffer

solution. A 20 μl aliquot of this suspension was used to examine for peptidylprolyl *cis-trans* isomerase activity. A 90 μl aliquot was used to examine for protein kinase activity.

Determination of Peptidylprolyl cis-trans Isomerase Activity

Peptidylprolyl *cis-trans* isomerase activity is determined by monitoring the chymotryptic cleavage of a peptide that can only occur when the X-Pro bond is *trans* and not when it is *cis*. Reaction media contained 75 μM peptide substrate, Suc-Ala-Leu-Pro-Phe-pNA (N-carboxypropionyl-Ala-Leu-Pro-Phe-p-nitroanilide) and 10 μM α-chymotrypsin in 50 mM HEPES buffer (pH 7.4). Total volume was 2000 μl. A 100 μl aliquot of an individual Sephacryl S-300 column fraction or a 20 μl aliquot of a suspension of the immunoprecipitated, immobilized proteins was used. When hrFKBP12 was examined in parallel experiments, its concentration was 45 nM. Determinations were conducted at 10° (controlled to 0.1°) with magnetic stirring, using a Hitachi U-2000 Enzyme Kinetics Data System which monitored absorption at 410 nm continuously. The isomerization reaction was initiated by the rapid addition of the sample and data were collected for 90 to 150 s when the reaction was uninhibited and for up to 15 min when it was strongly inhibited. Data were transferred from the U-2000 using a program provided by Dr. Harry Schmus (Hitachi, Danbury, Ct.) and values for k_{obs} were calculated using a variation of EnzFitter (BIOSOFT , Cambridge, U.K.). The time period used for these calculations began at 8 to 10 s and spanned 14 to 18 s (7 to 9 half-lives) when examining the inositolphosphate-binding protein or 50-55 s (approximately 9 half-lives) when examining FKBP12. In both instances, a sampling interval of 0.1 or 0.2 s was used. Strongly inhibited reactions were followed for up to 900 s (approximately 7 half-lives), with a sampling interval of 1.5 s. Values obtained for k_{obs} were approximately 0.1 s^{-1} (k_c/K_m in the range of 2 x 10^6 M^{-1} s^{-1}) for FKBP12 and approximately 0.35 s^{-1} (k_c/K_m in the range of 1 x 10^9 M^{-1} s^{-1}) for the inositolphosphate-binding immunophilin. For the non-enzymatic isomerization, k_{obs} was of the order of 0.004 s^{-1}.

Results and Discussion

Identification of an Inositolphosphate-binding Protein

Human erythrocytes, isolated by hypotonic lysis, were solubilized using Nonidet P-40 and subjected to gel filtration on Sephacryl S-300. When individual column fractions were examined for IP_3 binding using [5-^{32}P]IP_3 with the PEG precipitation procedure described by Chadwick et al. (9), fractions #41-#50 appeared to contain IP_3-binding proteins. Peak fractions and those adjacent to them were then combined in groups, concentrated 6-fold, and aliquots were examined by means of photoaffiniity-labeling studies using an arylazido derivative of an IP_3 or IP_4 analogue (8). The photoaffinity-labeling probe was a generous gift from Dr. Clinton E. Ballou. When the proteins that were labeled by the cross-linking reaction were examined on SDS polyacrylamide gels, a single, strongly labeled 12 kDa protein was identified in the case of Sephacryl S-300 column fractions #41-#50. There was no unequivocal indication of an inositolphosphate-binding protein in the other preparations. These data suggested that the human erythrocyte membrane contains a 12 kDa IP_3- and/or IP_4-binding protein.

The 12 kDa Protein Undergoes Phosphorylation and Is Co-immunoprecipitated with Anti-phosphotyrosine

When it was found that a 12 kDa protein could be labeled by both the ^{125}I-labeled arylazido inositolphosphate analogue and by [γ-^{32}P]ATP (with Mg^{2+} or Mn^{2+}), it was indicated that the 12

kDa inositolphosphate-binding protein can undergo phosphorylation. When this phosphorylation reaction was examined it was found that the phosphorylated 12 kDa protein was associated with two other phosphorylated proteins whose apparent masses were 30 and 38 kDa. Both phosphoamino acid analyses and immunoblots prepared with anti-phosphoserine or anti-phosphotyrosine indicated that the 12 kDa protein underwent phosphorylation on serine and that the associated 30 and 38 kDa proteins each underwent phosphorylation on both serine and tyrosine (data not shown). Because of the seemingly specific association between the 12 kDa protein and the 30 and 38 kDa proteins, a monoclonal antibody against phosphotyrosine was used to co-immunoprecipitate the three proteins. When the immunoprecipitated, immobilized proteins were examined, it was found that IP_3 binding could be demonstrated for the associated proteins, using the procedure described by Maranto (10), and that the 12, 30, and 38 kDa proteins each underwent phosphorylation. These observations suggested that the 12 kDa inositolphosphate-binding protein had been co-immunoprecipitated with a 30 and a 38 kDa protein, one or both of which might be a protein kinase.

The 12 kDa Protein Is Recognized by Anti-FKBP12

The indication that the 12 kDa inositolphosphate-binding protein is associated with a protein kinase activity suggested that there could be a similarity between it and immunophilins of the FKBP class which have been reported to associate specifically with phosphoryl-transfer enzymes including protein kinases, a phosphoprotein phosphatase, and a putative phosphatidylinositol kinase (11-20). We, therefore, examined preparations of the 12 kDa inositolphosphate-binding protein for homology with FKBP12, using immunoblots prepared with antibodies against FKBP12. The antibodies were a generous gift from Dr. Andrew R. Marks. These immunoblots identified a 12 kDa protein that was present specifically in Sephacryl S-300 column fractions or immunoprecipitated preparations that displayed IP_3-binding activity. This is shown in Figure 1, which also shows the expected strong signal from human recombinant FKBP-12. hrFKBP12 was a generous gift from Dr. Stuart L. Schreiber. It is suggested that the signal representing the inositolphosphate-binding protein (IPBP) is relatively weak because the protein being examined is phosphorylated and possibly lipid-conjugated (data not provided here) whereas the antigen, FKBP12, was not.

The 12 kDa Protein Has Peptidylprolyl *cis-trans* Isomerase Activity

The immunophilins display a characteristic peptidylprolyl *cis-trans* isomerase activity that is strongly inhibited by the appropriate immunosuppressant drug (21-25). We, therefore, examined preparations containing IP_3-binding activity and found that both individual Sephacryl S-300 column fractions and immunoprecipitated, immobilized preparations of the 12 kDa

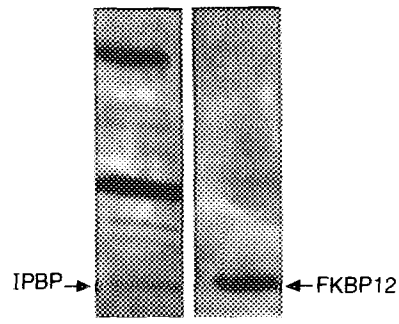

Figure 1. *Immunoblots prepared with anti-FKBP12.* An immunoprecipitated, immobilized preparation of the 12 kDa inositolphosphate-binding protein was resolved on an SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Hybond-ECL) that was then over-layed with polyclonal anti-FKBP12 (1:10,000). Immunoreactive proteins were detected by means of enhanced chemiluminescence, using a horseradish peroxidase-conjugated secondary antibody and luminol. (The very prominent signals in the panel on the left are due to the murine Ig light and heavy chains which are seen because the sample had been immunoprecipitated with monoclonal anti-phosphotyrosine.)

inositolphosphate-binding protein displayed robust peptidylprolyl *cis-trans* isomerase activity. Values for k_{obs}, the (pseudo) first order rate constant, were virtually the same for the two types of preparations and were 3.4 to 3.9 times the values we obtained for hrFKBP12 in parallel determinations. When the effects of the immunosuppressant drugs were determined, it was found that FK506 and rapamycin inhibited the peptidylprolyl *cis-trans* isomerase activities of the 12 kDa inositolphosphate-binding protein and FKBP12 to a similar extent, suggesting that the 12 kDa inositolphosphate-binding protein is an immunophilin of the FKBP class. When effects of IP_3 and IP_4 upon peptidylprolyl *cis-trans* isomerase activities were determined, it was found that nanomolar concentrations of each inhibited the activity of the membrane-associated inositolphosphate-binding immunophilin. However, neither IP_3 nor IP_4 inhibited the activity of the cytosolic FKBP12. Inositol 1,3,4-trisphosphate (at concentrations up to 100 nM) failed to inhibit under any of the conditions that were used. Selected values for k_{obs} are provided in Table 1.

IP_3 and IP_4 Binding

The binding of IP_3 or IP_4 by the 12 kDa inositolphosphate-binding immunophilin was examined using a nonlinear regression analysis (Graph Pad Prism) and samples of concentrated Sephacryl S-300 column fractions. A K_d value of 95.6 ± 7.2 nM, with B_{max} = 31.6 pmol/mg protein, was obtained for IP_3 binding. A K_d value of 14.4 ± 0.6 nM, with B_{max} = 13.9 pmol/mg protein, was obtained for IP_4 binding.

In summary, a 12 kDa (human) erythrocyte membrane protein that binds IP_3 or IP_4 has been identified and found to have the characteristics of an immunophilin of the FKBP class. In addition to FK506 and rapamycin, the peptidylprolyl *cis-trans* isomerase activity of this 12 kDa

Table 1

Peptidylprolyl *cis-trans* Isomerase Activity of the 12 kDa Inositolphosphate-Binding Immunophilin

	k_{obs} (s^{-1})		
No inhibitor	$0.3604 \pm 2 \times 10^{-4}$	No inhibitor	$0.3888 \pm 8 \times 10^{-4}$
1.0 nM rapamycin	$0.0054 + 2 \times 10^{-5}$	1.0 nM FK506	$0.0051 \pm 1 \times 10^{-5}$
1.0 pM rapamycin	$0.2933 + 2 \times 10^{-4}$		
No inhibitor	$0.3830 \pm 6 \times 10^{-4}$	No inhibitor	$0.3484 \pm 2 \times 10^{-4}$
1.0 nM IP_4	$0.0048 \pm 1 \times 10^{-5}$	20.0 nM IP_3	$0.0041 \pm 1 \times 10^{-5}$
1.0 pM IP_4	$0.2912 \pm 4 \times 10^{-4}$	1.0 pM IP_3	$0.2184 \pm 3 \times 10^{-4}$

inositolphosphate-binding protein is strongly inhibited by nanomolar concentrations of IP_3 or IP_4. Endogenous ligands for the immunophilins have not been identified previously and it is suggested here that IP_3 and IP_4 may be endogenous ligands for this membrane-associated immunophilin of the FKBP class.

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