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Functional Expression and Properties of Sec14p-Like Protein with Molecular Mass 45 kD from Rat Olfactory Epithelium

V. V. Radchenko[1]*, M. I. Merkulova[1], T. M. Shuvaeva[1], T. N. Simonova[1], A. A. Bondar[2], and V. M. Lipkin[1]

[1]Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya 16/10, 117997 Moscow, Russia; fax: (7-095) 335-7103; E-mail: radchenko@mail.ibch.ru
[2]Institute of Cell Biophysics, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia; fax: (7-096) 779-0509; E-mail: novoselov@mail.icb.psn.ru

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Abstract—cDNA of Sec14p-like water-soluble protein with molecular mass 45 kD from rat olfactory epithelium was expressed in *Escherichia coli* Rosetta™ cells. The expression product was purified by a two-step chromatographic procedure on DEAE-Sepharose and Sephacryl S-200. The identity of structural and functional characteristics of the recombinant and native proteins was demonstrated by CD, mass spectrometry, and Western blotting. Using several lipids immobilized on nitrocellulose membranes, it was shown that phosphatidylinositol-3,4,5-triphosphate is the specific ligand for the studied protein.

Key words: lipid-binding protein p45, olfactory epithelium, Sec14p family, CRAL/TRIO domain, protein–lipid interaction, phosphatidylinositol-3,4,5-triphosphate

A new protein with molecular mass 45 kD (p45) was isolated from rat olfactory epithelium several years ago [1]. A cDNA fragment encoding full-length p45 was obtained and its nucleotide sequence determined [2]. Analyzing the primary structure of p45, a conservative site containing ~250 amino acid residues was found; this site is typical of many lipid-binding/carrier proteins. Since this domain was first identified in SEC14 yeast protein (Sec14p), proteins containing this domain are ascribed to the Sec14p family [3, 4]. The domain is named SEC14 (smart00516 in Smart Data Base) or CRAL/TRIO (for retinaldehyde-binding proteins) (pfam 00650 in Pfam Data Base).

By now, more than 500 members of this evolutionarily ancient family have been found in protists, plants, yeast, invertebrates, and mammals [3]. The structures and functions of some proteins belonging to this family are well studied. Sec14p-like proteins participate in lipid transport, signal transduction, secretion regulation, and interaction of cellular compartments [4-9].

Although lipid-binding domains are very conservative, in each case the ligand specificity of proteins is defined by the polar part of the lipid molecule. Proteins of this family are known to bind trans-retinaldehyde [5], phosphatidylcholine [6], phosphatidylinositols [4], α-tocopherol [7], squalene [8], phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3) [9], etc.

Sec14p-like squalene-carrier protein isolated from bovine liver (SPF) is to a high extent homologous to the studied protein [8]. A corresponding human protein was cloned [7] and named tocopherol-binding protein (TAP), because its ability for binding to α-tocopherol was also demonstrated. It has been found that the human genome includes three TAP-like genes: *hTAP1*, *hTAP2*, and *hTAP3*. They are located on chromosome 22q12.1 not farther than 100 kb from each other and are 80-86% homologous [10]. The studied protein is homologous to human hTAP2 protein.

No natural ligands for TAP2 and TAP3 are known, and the data on ligand specificity of hTAP1/SPF are contradictory. As reported earlier, this protein is capable of binding to α-tocopherol [7, 11] and squalene [8]. While studying the 3D structure of hTAP1/SPF complex with α-tocopherols, it was found that the ligand molecule is located in a hydrophobic pocket formed by a site of amino acid sequence identified as the CRAL/TRIO domain [12]. Recently is was shown that recombinant hTAP1/SPF can bind to α-, β-, γ-, and δ-tocopherols, α-tocopheryl, squalene, phosphatidylcholine, and phosphatidylinositides [13]. The lack of a definite ligand pref-

* To whom correspondence should be addressed.

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erence of TAP-group proteins complicates the determination of the true native substrate.

Thus, the goal of the present work was to study the ligand specificity of p45 from rat olfactory epithelium.

MATERIALS AND METHODS

Reagents. In this study we used SDS, $(NH_4)_2SO_4$, Tris, Tween-20, ampicillin, chloramphenicol, rifampicin, BSA, Bromophenol Blue, ethidium bromide, and Brilliant Blue R from Sigma (USA); isopropyl-β-D-thiogalactopyranoside (IPTG) from MBI Fermentas (Lithuania); trypsin, yeast extract, and bactoagar from Difco (USA); Taq polymerase, *Nde*I and *Bam*HI restriction endonucleases, T4 phage DNA ligase, calf intestinal alkaline phosphatase, and Wizard Plus Minipreps DNA Purification System from Promega (USA); UltraClean DNA Purification Kit from MolBio Laboratories (USA); Protein WM molecular mass marker, DEAE Sepharose and Sephacryl S-200 from Pharmacia (Sweden); Immobilon-NC membrane from Millipore (USA); nitrocellulose filters with applied lipid sets PIP-Strips™ and PtdIns(3,4,5)P_3 from Echelon (USA); secondary rabbit antibodies conjugated with horseradish peroxidase from Sigma.

Bacterial strains. XL1 blue *E. coli* from Novagen (USA) was used for production of plasmid DNA and transformation on cloning. *Escherichia coli* Rosetta™ from Novagen was used for expression of cDNA cloned into the vector under the control of T7 phage promoter [14]. The strain contains the gene of T7 phage RNA polymerase controlled by inducible *lac*-promoter and plasmid with selective chloramphenicol-resistance marker; this marker controls synthesis of tRNA specific for AUA, AGG, AGA, CUA, CCC, and GGA codons.

Isolation and purification of p45 from rat olfactory epithelium. Wistar rats (50) pre-narcotized with chloroform were decapitated and olfactory epithelium was isolated and homogenized on an ice bath in 30 mM Tris-HCl buffer, pH 7.4, containing 145 mM NaCl using a manual Potter homogenizer. After centrifugation at 12,000*g* for 15 min, the supernatant was used for isolation of the protein. Purification included two sequential chromatographic separations on DEAE-Sepharose and Sephacryl S-200, as described in [1]. The final yield of protein was 0.5-0.7 mg from 50 rats. The epithelial tissue residues were used for total lipid isolation according to Folch [15].

Cloning of cDNA of rat p45. Recombinant DNA was processed according to the standard procedure [16]. cDNA fragment of rat p45 intended for cloning with retained reading frame in the expressing vector was obtained by polymerase chain reaction (PCR); oligonucleotides with inserted point replacements for obtaining corresponding restriction sites were used as primers. 5′-GT CTC AGC CAT **ATG** AGT GGC CGA G-3′ was used as a direct primer (site of cleavage by *Nde*I restrictase is underlined, the starting ATG codon is highlighted in bold) and 5′-G GGG ATC CAC **CTA** GAT AGG GGT GAG C-3′ was used as a reverse primer (site of *Bam*HI restrictase is underlined, the terminating TAG codon is highlighted in bold). pSP65 plasmid containing the cDNA sequence of rat p45 (GenBank™ AJ132352) was used as a template for PCR. This construction was earlier obtained in our laboratory by screening the phage library from rat olfactory epithelium cloned into the λNM1149 vector [2]. The reaction mixture (25 μl) contained ~1 ng of plasmid DNA, 15 pmol of each primer, 2.5 μl of tenfold buffer from Promega, 100 μM of each dNTP, and 2 U Taq polymerase. PCR was performed as follows: predenaturation (94°C, 5 min), then 30 cycles consisting of denaturation (94°C, 30 sec), annealing with primers (60°C, 30 sec) and elongation (72°C, 60 sec), and final construction of PCR products (72°C, 5 min). After treatment with the corresponding restrictases, the cDNA fragment of rat p45 was cloned in pET11-a(+) plasmid from Novagen via *Nde*I-*Bam*HI sites.

Expression of cDNA of rat p45. Transformation of competent *E. coli* Rosetta™ cells by the obtained plasmid pET11-a(+)/*p45-rat* was performed according to the standard procedure [16]. For production of recombinant protein, 1/100 of total cultivation volume of fresh overnight culture of *E. coli* Rosetta™/pET11-a(+)/*p45-rat* was applied in rich ×2YTG-M9 medium (standard ×2YTG medium containing 16 g/liter tryptone, 10 g/liter yeast extract, and 5 g/liter NaCl with addition of 6 g/liter Na_2HPO_4, 3 g/liter KH_2PO_4, 1 g/liter NH_4Cl, 2 mM $MgSO_4$, and 1 mM $CaCl_2$, pH 7.4). Cells were grown at 37°C in the presence of 35 μg/ml chloramphenicol and 100 μg/ml ampicillin until optical absorption A_{600} of liquid culture attained the value 0.6. To induce expression, IPTG was added to the final concentration 0.6 mM and cell growth continued for 60 min at 30°C. Then rifampicin was added to final concentration $5 \cdot 10^{-3}$ μg/ml to block translation of prokaryotic proteins. Cultivation was continued for 8-10 h at 28-30°C.

Electrophoresis. Cells of 1 ml culture were precipitated by centrifugation at 12,000*g* for 5 min at room temperature, the pellet was resuspended in 100 μl of buffer solution (45 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.01% Bromophenol Blue, 50 mM dithiothreitol (DTT)), heated for 10 min at 90°C, and samples 10-20 μl in volume were applied on the gel.

SDS-PAGE was performed in 12.5% polyacrylamide gel according to Laemmli [17]. Gels were stained in solution of 0.3% Brilliant Blue R and 10% acetic acid in 25% aqueous methanol. Unbound dye was removed by washing with 10% acetic acid in 25% aqueous methanol. A Protein WM kit was used as molecular mass marker.

Isolation and purification of recombinant p45. For preparative production of protein, the culture volume was

increased to 500-1000 ml. Cells were precipitated by centrifugation at 4000*g* for 20 min at 4°C, the pellet was resuspended on ice in PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.3), taking 50 μl of buffer per ml of cell culture. The suspension was frozen and then thawed and ultrasonicated on ice (power 200-300 Wt, 5 × 1 min with 1 min intervals). The suspension was centrifuged at 17,000*g* for 25 min at 4°C. Further procedures were performed at 4°C. To the supernatant dry $(NH_4)_2SO_4$ was added to the final concentration 2.0 M, the precipitate was removed by centrifugation at 12,000*g* for 15 min, the pellet was dissolved in 12 mM Tris-HCl, pH 7.8, and dialyzed against the same buffer containing 1 mM $MgCl_2$ and 1 mM DTT.

Dialyzed protein solution was chromatographed on DEAE-Sepharose in linear NaCl gradient (0-500 mM), as described earlier for the native rat protein [1]. The fractions were analyzed by SDS-PAGE. The recombinant p45 was then purified by gel filtration on Sephacryl S-200 in 25 mM Tris-HCl, pH 7.8, containing 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT. The procedure for p45 isolation is described in detail in [1].

Western blotting. Before application on Immobilon-NC nitrocellulose membrane from Millipore, proteins were separated by SDS-PAGE. Electroblotting was performed in buffer solution containing 20 mM $NaHCO_3$, pH 8.5, and 10% methanol at 75 mA for 50 min using a 2051 Midget Multiblot from LKB (Sweden). To prevent nonspecific sorption of primary antibodies, membrane was then incubated in TBS + Tween-20 buffer solution (50 mM Tris-HCl, pH 7.0-8.0, 150 mM NaCl, and 0.5% Tween-20) containing 3% BSA and 20% (v/v) glycerol for 1.5 h at 37°C. The membrane was washed thrice with TBS + Tween-20. The membrane was incubated with primary antibodies for 1 h at 30°C. Polyclonal rat antibodies used for incubation were obtained for native rat p45 in 1 : 750 dilution in TBS + Tween-20 containing 3% BSA and 20% (v/v) glycerol. Further steps were performed according to [18]. Secondary antibodies were detected by chemical luminescence using an ECL kit from Amersham Biosciences (England).

Circular dichroism was measured under non-denaturing conditions (25 mM Tris-HCl, pH 7.8, 100 mM NaCl) using a J-500A spectrometer from Jasco (Japan). Calculating molecular ellipticity per amino acid residue, the molecular mass of the latter was taken as 115 kD. The secondary structure was calculated according to Provencher [19].

Mass-spectrometry. A Vision 200 mass-spectrometer with time-of-flight base from Thermo Bioanalysis Corp. (USA) was used. The spectrometer was equipped with a pulsed nitrogen laser of 3B class (wavelength 337 nm (3.68 eV) with pulse frequency and duration 10 Hz and 3 nsec, respectively). Proteins were diluted in 50% trifluoroacetic acid, sample volume 5 μl at concentration ~1 mg/ml.

Lipid-binding specificity of p45 was studied according to Deaka et al. [2]. Lipids and/or total lipid extract isolated from olfactory epithelium according to Folch [15] and dissolved in a mixture chloroform–methanol–water (1 : 2 : 0.8 v/v) were applied onto a Hybond-C Extra nitrocellulose filter from Echelon. Samples were dried in air for 1 h at room temperature. To prevent nonspecific protein sorption, the membrane was treated by blocking buffer solution (TBS + Tween-20 containing 3% BSA free of fatty acids) for 2 h at room temperature. Then the membrane was put in fresh buffer solution of the same composition containing the studied protein at concentration 10 and/or 50 nM and incubated with stirring overnight at 4°C. The membrane was washed with TBS + Tween-20 mixture (three times for 10 min) and treated with primary polyclonal antibodies to p45 (1 : 750 dilution) at 37°C. Then membrane was washed thrice with TBS + Tween-20 mixture and treated with secondary anti-rabbit antibodies conjugated with horseradish peroxidase (1 : 5000 dilution) for 1 h at 37°C. Lipid-bound p45 was detected by chemiluminescence using an ECL kit from Amersham.

The same method was applied working with commercial PIP-Strips™ nitrocellulose membranes from Echelon with phospholipids applied onto them (100 pmol each per point).

RESULTS AND DISCUSSION

The data in the literature on ligand specificity of Sec14-like proteins are ambiguous. Even for one and the same protein first identified as squalene-carrier protein from rat liver but obtained in various recombinant systems, various substances were detected as ligands [7, 8, 11, 12]. Thus, to compare the structural and functional properties of recombinant and native p45 proteins, it was necessary to use the vector and conditions for obtaining recombinant preparation so that at least the primary structures of the recombinant and native proteins were completely identical.

Full-length cDNA of the water-soluble protein with molecular mass 45 kD from rat olfactory epithelium was cloned into the pET11-a(+) vector via *Nde*I-*Bam*HI restrictase sites. For further work, we chose pET11-a(+)/*p45-rat* plasmid containing an open reading frame controlled by T7 phage promoter; this reading frame included initial ATG codon, cDNA of p45 rat protein, and TAG terminating codon.

Our attempts to express cDNA of p45 rat protein in prokaryotic systems (in *E. coli* BL21(DE3) and B834(DE3) strains) under the standard conditions were unsuccessful. Analysis of nucleotide sequence of cDNA of p45 rat protein revealed multiple triplets corresponding to tRNA rarely present in bacterial cells but often found in high organisms. This seems to be the main reason that

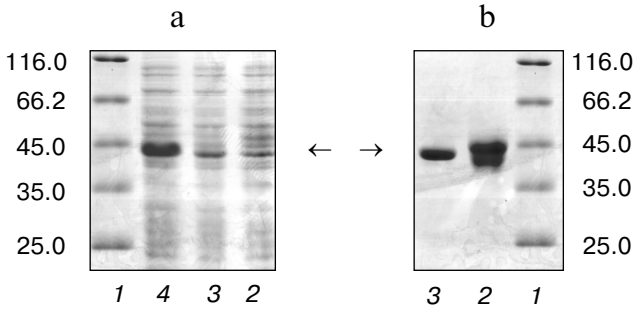

Fig. 1. Analysis of expression of cDNA of rat p45 protein (a) and steps of its chromatographic purification (b) by the SDS-PAGE in 12.5% polyacrylamide gel. a) Total *E. coli* Rosetta™ cell proteins bearing the initial pET11-a(+) plasmid (*2*) and pET11-a(+)/*p45 rat* plasmid before induction by IPTG (*3*) or 6 h after induction by IPTG (*4*); *1*) protein molecular mass markers (kD). b) Recombinant p45 protein after chromatography on DEAE-Sepharose (*2*) and after subsequent chromatography on Sephacryl S-200 (*3*); *1*) protein molecular mass markers (kD). The position of the recombinant protein is shown by arrows.

in the cell the studied protein is synthesized in low quantities. Thus, for expression of p45 cDNA we chose *E. coli* Rosetta™ from Novagen having the gene of T7 phage RNA polymerase controlled by inducible *lac* promoter in its chromosome. Cells of this strain bear a plasmid having a selective resistance marker against chloramphenicol and controlling synthesis of tRNA specific for AUA, AGG, AGA, CUA, CCC, and GGA codons.

The resulting pET11-a(+)/*p45-rat* plasmid was used for transformation of *E. coli* Rosetta™ cells. Expression products and the degree of sample purity after chromatography were analyzed by SDS-PAGE (12.5% polyacrylamide gel) (Figs. 1a and 1b).

Recombinant protein with the expected molecular mass was found in the fraction of water-soluble proteins obtained after ultrasonic cell disintegration. Results of electrophoretic analysis of water-soluble proteins from cell lysate before and after addition of IPTG are presented in Fig. 1a. After IPTG induction of expression, protein with molecular mass ~45 kD was intensively produced (lanes *2-4*). Densitometry of the stained polyacrylamide gel showed that the yield of this protein was ~5 mg per liter of culture.

To prove that this protein is a recombinant analog of rat p45, we performed Western blotting using polyclonal rabbit antibodies to the native p45 protein (Fig. 2). The data indicate that the protein with molecular mass ~45 kD, whose concentration drastically increased in *E. coli* cells after addition of IPTG, is lipid-binding p45 rat protein.

For further study, p45 was obtained in preparative quantities. Protein was purified by chromatography on DEAE-Sepharose and gel filtration on Sephacryl S-200 according to the procedures developed earlier for native rat protein [1, 2]. The yield of electrophoretically pure protein was ~2 mg per liter of culture. The purified preparation was a water-soluble protein with electrophoretic mobility corresponding to a polypeptide with molecular mass ~45 kD (Fig. 1b).

For manifestation of biological activity of protein, proper formation of its secondary and tertiary structure is necessary. Thus, CD spectra of recombinant p45 and protein isolated from rat olfactory epithelium were recorded under the non-denaturing conditions (Fig. 3); both polypeptides appeared to have distinct secondary structure. The experimental spectra were resolved into α-helix, β-sheet, and irregular components according to Provencher [19]. Since the natural and recombinant p45 proteins are composed of nearly equal portions of α-helices (50%) and β-sheets (20%), the recombinant protein seems to adopt a conformation close to that of the native protein.

To obtain information about post-translational protein modifications, the mass spectra of recombinant p45 and protein from rat olfactory epithelium were compared. For the native protein, the $[M + H]^+$ value was 44,656 (Fig. 4a), whereas for the recombinant protein this value was lower by 91 daltons (44,565) (Fig. 4b). Since the PNPDD*Y*FLLRWLRARNF sequence (31-47), a potential site of action for tyrosyl protein sulfotransferases [21], is a constituent of p45 molecule, we suggest that the Y36 residue, a constituent of the above mentioned sequence, is sulfonated in the native protein. Such a site is known for three proteins of this family (*hTAP1*, *hTAP2*, and *hTAP3* gene products) and is located in the CRAL/TRIO domain. The role of this post-translational modification is very important in the protein–protein interactions, protein–membrane associations, and in cell secretory

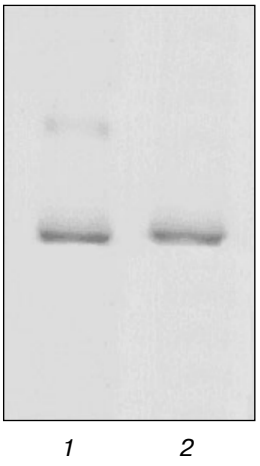

Fig. 2. Immunochemical identification of recombinant p45 (*1*) and protein isolated from rat olfactory epithelium (*2*) using polyclonal antibodies to p45 rat protein. Dilution of primary antibodies 1 : 750, secondary 1 : 5000. Detection by enzyme chemiluminescence (ECL).

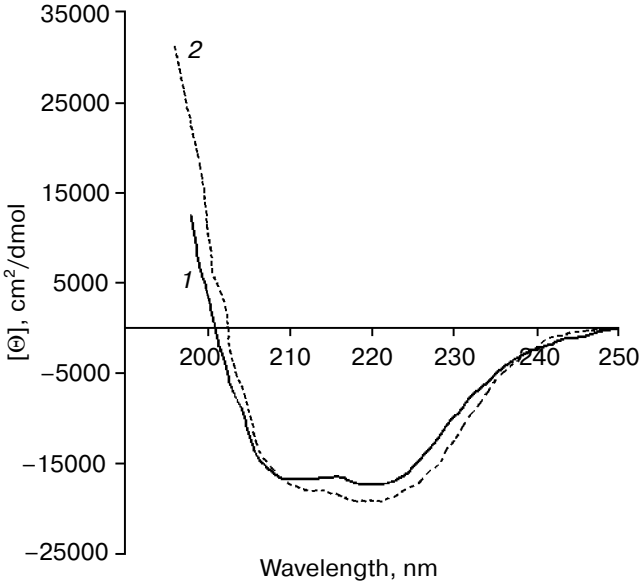

Fig. 3. CD spectra of protein isolated from rat olfactory epithelium (*1*) and recombinant p45 protein (*2*) recorded under nondenaturing conditions.

pathways [22]. However, the difference in the molecular masses of the studied samples (taking the molecular mass of one sulfo group as 80 daltons) cannot be explained by this modification alone.

A cDNA fragment encoding the native protein provides for synthesis of the 400-mer polypeptide chain bearing an N-terminal methionine residue. As it was found earlier on determination of p45 primary structure, the N-terminal residue of p45 is blocked [2]. The data suggest that excision of the N-terminal methionine residue and subsequent modification of the penultimate serine residue occurs during p45 polypeptide chain processing. In this case, the molecular mass of the modifying group should be 223 daltons (91 + 132 (molecular mass of the methionine residue)). The myristic acid residue with molecular mass 211-215 daltons (depending on isotope content) is the best to fit these data. Myristoylation via the N-terminal Gly residue is typical of many G-proteins [23, 24] including the Rab-family of minor G-proteins, and human TAP are representatives of the latter [25]. But the consensus sequence necessary for activity of the known N-myristoyltransferases [26] is not observed in the studied protein. Possible post-translational TAP modification was not examined earlier; detection of the modifying group of p45 allows a new look at the functional role of the studied protein.

As mentioned above, in certain cases substrate specificities of recombinant forms obtained in various expressing systems differed from each other [8, 9, 11, 12]. Thus, the specificity of p45 binding was studied simultaneously for the native and recombinant proteins.

Squalene and α-tocopherol are well-known ligands for some proteins belonging to Sec14p family [7-11]. The p45 protein also belongs to this family, so its capacity to bind to α-tocopherol and squalene was studied. As found, p45 interacts with squalene and total lipid extract from epithelium lining (Fig. 5). Fractioning of the extract by TLC gave nine distinct lipid zones [27]. Lipids of each zone were extracted and their capacity to bind to p45 was studied. Since mobility of the lipid fraction interacting with p45 significantly differed from that of squalene (R_f = 0.3 and 0.9, respectively, data not presented here) and qualitative reaction of this lipid for phospholipids was positive [27], at least one more substance specifically interacting with the studied protein should be present in the lipid extract.

Phospholipid ligand for p45 was identified using a PIP-Strip™ membrane from Echelon; almost all phospholipids found in eukaryotic cells are applied onto this membrane (100 pmol of individual substance per point). For removal of nonspecific signals, we treated membranes with solutions containing natural and recombinant proteins at two concentrations, 50 and 10 nM (Figs. 6a and 6b). In both cases p45 showed the highest affinity to PtdIns(3,4,5)P_3. However, it should be mentioned that at high protein concentration all phosphatidylinositols gave

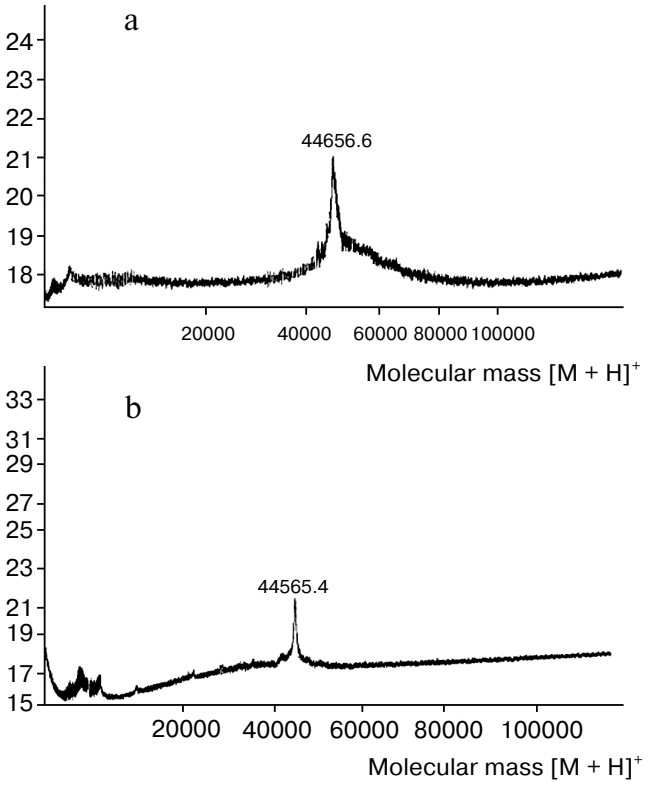

Fig. 4. Estimation of molecular mass by mass-spectrometry. Protein from rat olfactory epithelium (a) and recombinant p45 protein (b).

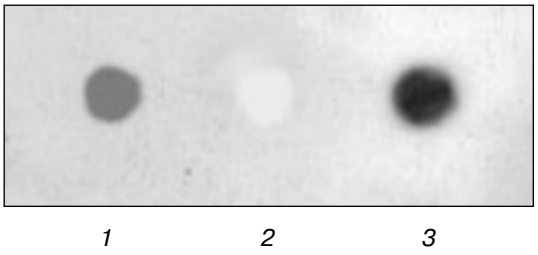

Fig. 5. Binding of p45 protein (concentration 30 nM) from rat olfactory epithelium to various hydrophobic ligands: *1*) squalene; *2*) α-tocopherol; *3*) total lipid extract from olfactory epithelium lining.

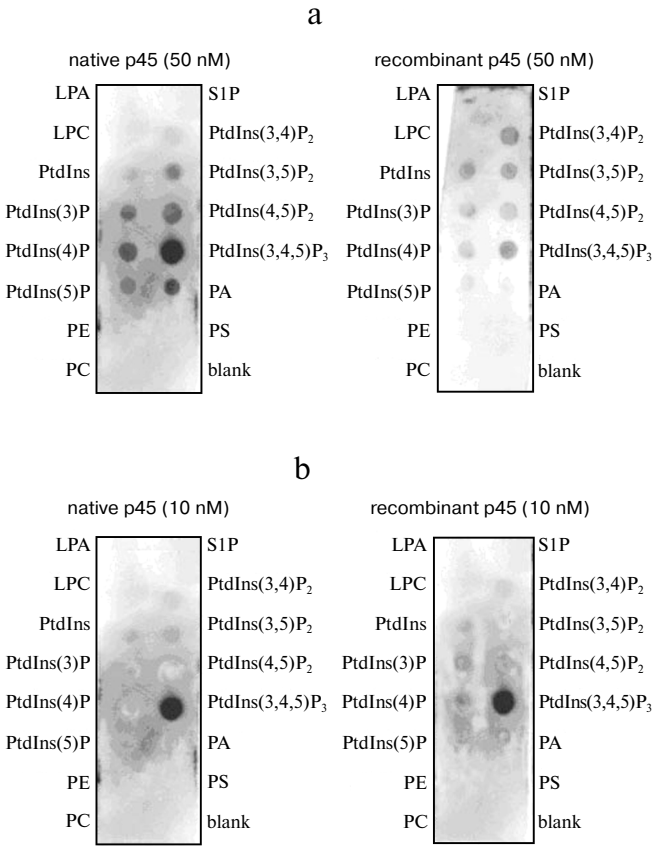

Fig. 6. Study of lipid-binding specificity of p45. PIP-Strips™ membranes from Echelon with lipid set treated with preparations containing native and recombinant proteins (two replicates). Protein concentrations (nM): a) 50; b) 10. Phospholipids (100 pmol each per point): phosphatidylinositol (PtdIns), phosphatidylinositol-3-monophosphate (PtdIns(3)P), phosphatidylinositol-4-monophosphate (PtdIns(4)P), phosphatidylinositol-5-monophosphate (PtdIns(5)P), phosphatidylinositol-3,4-diphosphate (PtdIns(3,4)P_2), phosphatidylinositol-3,5-diphosphate (PtdIns(3,5)P_2), phosphatidylinositol-4,5-diphosphate (PtdIns(4,5)P_2), phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P_3), phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), lysophosphatidic acid (LPA), lysophosphatidylcholine (LPC), sphingosine-1-phosphate (S1P), and phosphatidic acid (PA).

positive but rather weak signals. At decreased protein concentrations the interaction specificity increased (Fig. 6b), and both natural and recombinant proteins bound almost only to PtdIns(3,4,5)P_3. So, at least in the studied tissue (olfactory epithelium lining) PtdIns(3,4,5)P_3 is a natural ligand for p45.

Phosphatidylinositol PtdIns(3,4,5)P_3 is a key mediator in various signal pathways inducing and regulating such cell processes as differentiation, chemotaxis, environmental adaptation, glucose transport, and homeostasis [28]. The type and isoform of phosphatidylinositol-3-kinases (PI3Ks) are the main factors governing PtdIns(3,4,5)P_3 action because they provide accumulation of this phospholipid in cells. PI3Ks are classified depending on their localization, structure, and substrate specificity [29]. It is still not known whether PtdIns(3,4,5)P_3 binding to p45 is a participant of cascade of III PI3K located on intracellular membrane [30] or is synthesized by I PI3K [31] and then transported by p45 into Golgi apparatus and/or intercellular space. This will be a subject of our further investigations.

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