A Single Stranded DNA-Binding Protein, ssCRE-BP/Pur α , in Rat Lung and Its Increase in Allergic Airway Inflammation

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ABSTRACT—ssCRE-BP/Pur α is a single stranded DNA-binding protein and may be involved in gene replication and transcription and in the development of morphine dependence. We found a ssCRE-BP/Pur α (45 kDa) in rat lung that was larger than those (40 kDa) identified in rat and mouse brains and mouse lung. Immunohistochemistry showed that ssCRE-BP/Pur α is primarily distributed in the lung epithelium. As allergic inflammation induces various gene expressions, we investigated the changes of Pur α during airway inflammation. Ovalbumin-sensitized rats were used for inducing allergic airway inflammation. The expression and DNA-binding activity of 45-kDa ssCRE-BP/Pur α were significantly increased in the sensitized rat lungs 24 hr after antigen challenge, but not in those of rats nonsensitized or sensitized with ovalbumin and challenged with saline. Immunohistochemistry and *in situ* hybridization demonstrated that the vascular endothelial cells and numerous infiltrated eosinophils around the airways were stained with anti-Pur α antibody. These data suggest that rat lung and the eosinophils contain a 45-kDa ssCRE-BP/Pur α that is increased when airway inflammation occurs.

Keywords: Single stranded DNA-binding protein, ssCRE-BP/Pur α , Allergic airway inflammation, Eosinophil, Lung

Pur α is a single stranded DNA-binding protein and has a partial homology with several proteins involved in the initiation of replication or transcription, including SV40 large T antigen (1–3). Pur α binds in a sequence-specific manner to tandem repeats of the triplet GGN, which were identified in the origin of JC virus DNA (4, 5) and the 5'-flanking region of mouse myelin basic protein (MBP) (6, 7). Pur α has been reported as a replication or transcription regulatory factor in proeukaryotic and eukaryotic cells (8).

Recently, we have reported that the DNA-binding activity of a single stranded cyclic AMP response element-binding protein (ssCRE-BP) is decreased in mouse cerebella or in cultured NG108-15 cells after chronic morphine administration (9, 10), and the cDNA sequence of mouse ssCRE-BP is identical to mouse Pur α (11, 12).

Airway inflammation with eosinophil infiltration is a prominent feature of allergic respiratory diseases such as

asthma. The pathobiology of the pulmonary eosinophil is dependent upon factors that regulate eosinophilopoiesis, adherence, migration and activation of the eosinophils. Several cytokines such as interleukin 4 (IL-4), interleukin 5 (IL-5), tumor necrosis factor (TNF) and granulocyte macrophage-colony stimulating factor (GM-CSF) play important roles in controlling eosinophil function (13). Although the precise mechanisms of pulmonary infiltration of the pulmonary eosinophils are not clear, it has recently been demonstrated that the stimulation of transcription factors, such as nuclear factor- κ B (NF- κ B), activating protein-1 (AP-1) and nuclear factor of activated T cells (NF-AT), is a critical step in activating specific gene transcription to initiate the eosinophil infiltration and eosinophil-induced tissue damage (14–17). In this study, we found that a ssCRE-BP/Pur α in rat lung differs from that of other tissues in molecular size by Western blot analysis, and we investigated its changes in allergic airway inflammation.

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MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 150–170 g and male ddY mice weighing 25–30 g were supplied by Nihon Animal Co., Osaka. All laboratory animal care and treatment followed the guidelines of The Japanese Pharmacological Society.

Preparation of nuclear extract and gel shift assay

Crude nuclear extracts were prepared from rat or mouse lung and brain according to the method described previously (10, 11). The tissues were immersed into 1 ml of buffer A per 100 mg wet weight. Buffer A consisted of 0.25 M sucrose, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT) and 10 mM HEPES (pH 7.5). The tissues were homogenized by 10 strokes of a Teflon-pestled Potter homogenizer (Wheaton, Millville, NJ, USA), followed by centrifugation at $500 \times g$ for 10 min. The pellet was resuspended in the same volume of buffer A, and the suspension was centrifuged again as above. Then the pellets were suspended in buffer C containing 20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 mM DTT, 0.2 mM EDTA and 20% glycerol. The suspension was stirred gently at 4°C for 1 hr, and then the extract was centrifuged at $25,000 \times g$ for 20 min at 4°C to remove the debris. The supernatant was dialyzed against buffer D overnight. Buffer D consisted of 20 mM HEPES (pH 7.9), 100 mM KCl, 0.5 mM PMSF, 0.5 mM DTT, 0.2 mM EDTA and 20% glycerol.

Gel shift assays were performed as described previously (10, 11). Briefly, the reaction mixture containing 12 mM HEPES (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM DTT, 12% glycerol, 1 μg poly(dI-dC), ^{32}P -labeled ssCRE with or without the nuclear extracts was incubated at room temperature for 30 min. It was analyzed on 4% non-denaturing polyacrylamide gels. The sense strand of ssCRE in the rat somastatin (soma) promoter, 5'-CTGG GGGCGCCTCCTTGGCTGACGTCAGAGAGAGAG-3' (18) was end-labeled with [γ - ^{32}P]ATP and T4 polynucleotide kinase. In competition assays, an excess of unlabeled competitor oligo DNA was preincubated with the nuclear extracts for 10 min. For the supershift assay, 1 μl of rabbit polyclonal antibody against ssCRE-BP/Pur α was added into the reaction mixture and incubated for 30 min at 4°C before the addition of the probe.

Western blot analysis

Polyclonal antisera against ssCRE-BP/Pur α were raised in rabbits by immunization with glutathione *S*-transferase (GST)-ssCRE-BP/Pur α fusion proteins (11). A 15- μg sample of proteins from the lung or brain nuclear

extracts were separated by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a PVDF membrane (0.2 μm ; Bio-Rad, Hercules, CA, USA). The membrane was reacted with the polyclonal anti-ssCRE-BP/Pur α antisera. An ECL system (Amersham, Buckinghamshire, UK) was used for detection of the immunoreaction. To test the specificity of the anti-GST-ssCRE-BP/Pur α , 35 μg of GST-Pur α fusion protein was mixed with 5 μl of the antiserum at 4°C overnight to neutralize the antibody. Then it was centrifuged, and the resulting supernatant was used for Western blot analysis.

Immunization and challenge

Rats were sensitized by injecting them with a mixture of ovalbumin and aluminum hydroxide under anesthetization with intraperitoneal injection of pentobarbital (30 mg/kg). One hundred milligrams of aluminum hydroxide was added to the ovalbumin solution (1 mg/1 ml saline) (19). The solution was gently rotated for 60 min to obtain an aluminum hydroxide gel, and 0.5 ml was injected intraperitoneally, while another 0.5 ml was divided over 10 subcutaneous injection sites in the proximity of the lymph nodes in the paws, lumbar and neck regions. The nonsensitized animals were injected with aluminum hydroxide gel (100 mg in 1 ml saline) in the same way. After two weeks, the rats were anesthetized, the ventral surface of the neck was incised longitudinally for about 1 cm starting below the level of the thyroid cartilage, and the trachea was exposed. Ovalbumin solution (20 μg in 0.1 ml saline) or saline (0.1 ml) was injected into the trachea directly, together with 1 ml air to distribute the solution in the respiratory tract. After 24 hr, the animals were sacrificed and the lungs and brains were removed to prepare nuclear extracts. Some animals were perfused with saline and 4% phosphate-buffered paraformaldehyde for preparing tissue sections as described below.

Immunohistochemistry and histological examination

The rat was perfused with 60 ml saline followed by 300 ml 4% phosphate-buffered paraformaldehyde (pH 7.4). The lungs were isolated and fixed further with 4% paraformaldehyde at 4°C overnight. The fixed lungs were sectioned in a cryostat chamber. The sections (10- μm -thick) were incubated with ssCRE-BP/Pur α antibody (diluted at 1:2000) as previously described (11). Then the sections were washed and incubated with FITC-conjugated goat antibody against rabbit IgG (Cappel, Malvern, PA, USA; diluted at 1:1000). The sections were then examined and photographed under a fluorescence microscope (Zeiss, Oberkochen, Germany). The immunoreactive cells except for the epithelium and vessels were counted at $400 \times$ magnification in 5 randomly selected

areas surrounding the bronchi. After immunohistochemical examination, the lung sections were stained with hematoxylin and eosin. The number of eosinophils surrounding the bronchi were counted in the same areas observed in the immunostained sections at magnification $\times 400$.

In situ hybridization

For the template of RNA probe synthesis, the ssCRE-BP/Pur α cDNA was subcloned into a pBluescript II SK(-) vector and digested with restriction enzymes, EcoR1 and BamH1. Labeled sense and antisense cRNA probes were generated by using T3 and T7 RNA polymerases with digoxigenin-UTP according the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). The tissue sections were prepared as described above. The slides were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 20 min. Prehybridization and hybridization were carried out as described previously (20). After hybridization, the slides were washed by $5\times$ SSC at 50°C and treated with RNase A. The tissue sections were then incubated for 3 hr with a 1:1000 dilution of anti-digoxigenin Fab fragments conjugated with alkaline phosphatase (Boehringer Mannheim). The color reaction was stopped by washing after an overnight incubation in the presence of 4-nitro blue tetrazolium chloride (NTB).

Statistical analyses

Results were expressed as means $\pm$ S.E.M. The difference between two groups was determined by an unpaired *t*-test or the Mann-Whitney *U*-test as appropriate. Differences with $P < 0.05$ were judged significant.

RESULTS

Characterization of rat lung ssCRE-BP/Pur α

When the molecular size of ssCRE-BP/Pur α in rat lungs was compared to that in other tissues by Western blot analysis, we observed a major band of 45 kDa and several weak larger bands in rat lung (Fig. 1: lane 2), while rat and mouse brains and mouse lung contained a 40-kDa ssCRE-BP/Pur α protein (Fig. 1: lanes 1, 3 and 4). We carried out the following experiments. When ssCRE-BP/Pur α polyclonal antibody was absorbed with purified GST-ssCRE-BP/Pur α fusion protein at 4°C overnight, the neutralized antibody almost completely lost its reactivity to the 40- and 45-kDa proteins in the mouse and rat tissues (Fig. 1: lanes 5–8). Treatment with *N*-glycosidase F of the nuclear extract from rat lung did not reduce the molecular size of 45 kDa, indicating that 45-kDa Pur α is not glycosylated. Another antibody against ssCRE-BP/Pur α , which was produced at a separate time, also detected the 45-kDa protein in rat lung and 40-kDa proteins in the other tissues.

Fig. 1. Detection of Pur α in the lung and brain of rat and mouse by Western blot analysis. Nuclear extracts from the brains and lungs of rat and mouse were separated by 10% SDS-PAGE and then transferred to a PVDF membrane. Pur α was detected by a rabbit polyclonal antibody against GST-ssCRE-BP/Pur α fusion protein (1:1000). In lanes 5–8, the antibody was neutralized with GST-ssCRE-BP/Pur α fusion protein at 4°C overnight. Each lane was applied with 15 μg protein. Lanes 1 and 5: rat brain, 2 and 6: rat lung, 3 and 7: mouse brain, and 4 and 8: mouse lung.

In the gel shift assay, we prepared a [32 P]-labeled oligonucleotide probe of soma-ssCRE, a sense strand of the cyclic AMP response element (ssCRE) in the rat somastatin promoter (18). The nuclear extracts from the brains and lungs of rat and mouse formed a DNA-protein complex with the DNA probe, and it was migrated slowly (supershifted) by the addition of ssCRE-BP/Pur α antibody (Fig. 2A), although the DNA-protein complex and the supershifted band of rat lung showed slightly larger sizes than those of the other tissues (Fig. 2A). The DNA-binding activity increased with increasing amounts (1, 2.5 and 5 μ g protein) of rat lung nuclear extract (Fig. 2B: lanes 2–4). The formation of the DNA-protein complex was decreased by the addition of increasing unlabeled competitor of oligo DNA (Fig. 2B: lanes 5–7). These data suggest that rat lung contains a 45-kDa species that has the same DNA-binding properties as those of the 40-kDa protein.

Increases of Pur α expression and DNA-binding activity in the antigen-induced airway inflammation

The ovalbumin-sensitized rats were used to investigate the changes in a 45-kDa Pur α in antigen-induced airway inflammation. Rats were sensitized with ovalbumin, and challenged by intratracheal instillation with ovalbumin 2 weeks after sensitization. After 24 hr of the challenge with ovalbumin, the lung and brain nuclear extracts were prepared for detecting the expression of the Pur α and DNA-binding activity. Western blot analysis showed that the expression of Pur α was only increased in the sensitized rat lungs challenged with ovalbumin (Fig. 3: line 4), not in those of rats sensitized with ovalbumin and challenged with saline or nonsensitized and challenged with saline and ovalbumin (Fig. 3: lanes 1–3). The expression of brain Pur α was not significantly different in each group (Fig. 3: lanes 5–8). The DNA-binding activity in the gel shift assay was also increased in the sensitized rat lungs challenged with ovalbumin, but not in the others (Fig. 4: A and B).

Fig. 2. DNA-binding activity of the nuclear extracts from rat lungs. Nuclear extracts (N.E.) from lung and brain were incubated for 30 min with the [32 P]-labeled oligonucleotide (25 fmol), which was soma-ss-CRE, the sense strand of the cyclic AMP response element (CRE) in the rat somastatin promoter. Then the reaction mixture was electrophoresed in 4% non-denaturing polyacrylamide gels, and the gels were dried and autoradiographed. Panel A: Comparison of rat lung DNA-binding activity (lanes 3 and 7) with those of rat brain (lanes 2 and 6), mouse brain (lanes 4 and 8) and mouse lung (lanes 5 and 9). Each lane was applied with 5 μ g protein. Lanes 6–9 show the supershift assay using ssCRE-BP/Pur α antibody (Ab). Lane 1, no nuclear extract. Panel B: Binding activities of different amounts (0–5 μ g) of rat lung nuclear extract (lanes 1–4) and competition by unlabeled oligonucleotide (lanes 5–7).

Fig. 3. Increased expression of Pur α in sensitized rat lung after challenge with ovalbumin. Pur α in the nuclear extracts from rat lung (lanes 1–4) and brain (lanes 5–8) was detected by Western blotting with ssCRE-BP/Pur α antibody (1:1000). Nuclear extracts were from nonsensitized rats challenged with saline (lanes 1 and 5) or ovalbumin (lanes 2 and 6) and from sensitized rats challenged with saline (lanes 3 and 7) or ovalbumin (lanes 4 and 8). Fifteen micrograms of protein was added into each lane. Note the increased expression only in sensitized rat lung challenged with ovalbumin (lane 4).

Fig. 4. Enhancement of DNA-binding activity in sensitized rat lung after challenge with ovalbumin (OA). Panel A: Gel shift assay for the DNA-binding activity of Pur α with [³²P]-labeled oligonucleotide. The lung nuclear extracts were from nonsensitized rats challenged with saline (lane 2) or ovalbumin (lane 3) and from sensitized rats challenged with saline (lane 4) or ovalbumin (lane 5). Mouse brain nuclear extract (lane 6) was used as a positive control. Five micrograms of protein was applied in each lane. Lane 1 contained only the probe. Panel B: The summary of binding activities from 4 independent experiments. The radioactivity of the DNA-protein complex was measured using a Fujix Bio-imaging Analyzer BSA-2000 (Fuji Film, Tokyo) and was expressed as the percentage of the binding activity of mouse brain nuclear extract. Means $\pm$ S.E.M., $n=4$ in each group; * $P<0.05$, compared with each of the other groups (t -test).

Increased expression of Pur α and its mRNA in eosinophils and vascular vessels of the antigen-induced airway inflammation

Morphological examination revealed evidence of severe inflammation in the lungs of sensitized rats at 24 hr after they were challenged with ovalbumin. Numerous eosinophils, and a small number of mononuclear and polymorphonuclear cells were seen around the vessels and bronchi (Figs. 5B and 6B), confirming a characteristic eosinophilic airway inflammation induced by the specific antigen (13). Thickening of vascular walls and an infolding of the epithelium were also observed. The inflammation was more prominent in the area surrounding the larger bronchi and less in the alveolar regions. Eosinophil infiltration and other inflammatory evidence were hardly found in the lungs of sensitized rats challenged with saline (Figs. 5A and 6B) and in those of nonsensitized rats challenged with saline or ovalbumin (data not shown).

Pur α was detected in the bronchial epithelium and in some alveolar, mesenchymal and pericapillary cells in the

control lungs, but it was weaker or undetectable in the vessels, bronchial and vascular smooth muscular cells. The epithelial cells in the smaller bronchi (Fig. 5C) were immunostained more strongly than those in larger bronchi (Fig. 5D). In the inflamed lung sections, the infiltrated eosinophils were strongly stained and the staining of vascular endothelial cells was also observed (Figs. 5D and 6A). However, no enhanced immunostaining was observed in the bronchial epithelial cells. Figure 6 indicates that the increase of immunoreactive cell numbers was parallel with the increase of eosinophils. When non-immunized rabbit serum was used for immunohistochemical examination, no apparent immunoreactivity was observed in the lung sections of each group (data not shown).

Furthermore, we used a digoxigenin-labeled antisense cRNA probe to detect the expression and distribution of ssCRE-BP/Pur α mRNA in the lungs of each group. The *in situ* hybridization results showed the similar distribution pattern of ssCRE-BP/Pur α mRNA to that of the immunohistochemical staining, although it was more

Fig. 5. Histological and immunohistochemical examinations of sensitized rat lungs. Panels A and B are stained with hematoxylin and eosin. Panels C and D are the immunostaining with ssCRE-BP/Pur α antibody. Panels A and C are from sensitized rat challenged with saline, and Panels B and D are from rat challenged with ovalbumin. The arrowheads represent some immunoreactive eosinophils. The epithelial cells on the smaller bronchus (E in panel C) is immunostained more strongly than those on the larger one (E in panel D). V: vessel, E: epithelium. Magnification $\times 400$.

Fig. 6. Increases of Pur α -immunoreactive cells (A) and eosinophils (B) in the lungs of sensitized rats challenged with ovalbumin (OA). Panel A: The numbers of immunoreactive cells were summed from 5–7 experiments as shown in Fig. 5 (C and D). Means $\pm$ S.E.M., $n=5-7$; * $P<0.05$, compared with each of the other groups (t -test). Panel B: The numbers of eosinophils were summed from 5–7 experiments as shown in Fig. 5 (A and B). Means $\pm$ S.E.M., $n=5-7$; ** $P<0.01$, compared with each of the other groups (Mann-Whitney U -test).

Fig. 7. Expression of ssCRE-BP/Pur α mRNA in the sensitized rat lungs. *In situ* hybridization was carried out using a digoxigenin-labeled antisense ssCRE-BP/Pur α cRNA probe. Panels A and C show the result of a sensitized rat challenged with saline, and panels B and D are the results of a rat challenged with ovalbumin. Expression of ssCRE-BP/Pur α mRNA is observed in the infiltrated eosinophils (arrowheads in panel B), vascular endothelial cells (arrow in panel B), and small vessels (in panel D). Magnification $\times 400$.

sensitive than immunohistochemistry. The infiltrated eosinophils and endothelial cells of larger vascular vessels expressed ssCRE-BP/Pur α mRNA in the sensitized rat lungs (Fig. 7: B and D) much more strongly than the control rat lungs (Fig. 7: A and C). The sense cRNA probe did not elicit any specific hybridization signal (data not shown).

DISCUSSION

We found that ssCRE-BP/Pur α (45 kDa) in the rat lung is larger than those (40 kDa) in rat brain and mouse brains and lung by Western blot analysis. The following evidence supports that 45-kDa protein in rat lung is Pur α : 1) Rat lung does not contain any 40-kDa protein that reacts with Pur α antibody. 2) Another antiserum against ssCRE-BP/Pur α also detected the 45-kDa band in rat lung, but reacted with the 40-kDa band in rat brain. 3) Antibody adsorbed with ssCRE-BP/Pur α did not react with either the 45-kDa band in rat lung or the 40-kDa band in the other tissues as shown in Fig. 1. 4) Glycosidase treatment did not reduce the molecular size of the 45-kDa band. 5) Distribution of Pur α mRNA is well paralleled that of the protein. 6) The DNA-protein complex in the gel shift assay competed with cold DNA containing Pur α sequence and the band was supershifted by the addition of the antibody. These data indicate that the 45-kDa band of rat lung has the same properties as those of the 40-kDa protein in the other tissues (11, 12). Several weaker bands larger than 45 kDa in rat lung also disappeared when the antibody was preabsorbed with GST-Pur α (Fig. 1), suggesting the existence of precursor proteins, isoforms or post-translationally modified proteins such as the phosphorylated species. Recently an isoform of Pur α has been reported as Pur β in vascular smooth muscle (21). The cDNA sequence shows that there is a 71% amino acid homology between Pur α and Pur β . Although it is interesting that Pur α differs in molecular size between mouse and rat lungs, the physiological significance is unclear.

Since it is known that stimulation of transcription factors such as NF- κ B is a critical step in activating the specific gene transcription leading to eosinophil infiltration and eosinophil-induced tissue damage (14–17), we examined the changes in Pur α in airway inflammation. The animals were challenged by intratracheal instillation of antigen in this study to keep the antigen in the airway for a longer duration and application of a more precise amount. A significant eosinophil infiltration surrounding the bronchi was observed in the sensitized rat lungs at 24 hr after the antigen challenge when the peak of eosinophilia is observed in antigen-induced airway inflammation of guinea pigs (22). The expression and DNA-

binding activity of Pur α were increased only in the inflamed lungs of sensitized and challenged rats. Immunohistochemistry and *in situ* hybridization showed that the increased expression of Pur α was found mainly in the infiltrated eosinophils and the endothelial cells of larger vascular vessels, whereas it was distributed primarily in the bronchial epithelium in control rat lung. In another study, we have found that transplanted rat kidney exhibited the increased infiltration of lymphocytes that express 40-kDa ssCRE-BP/Pur α in the acute phase of the rejection reaction (unpublished data). Eosinophils and vascular endothelial cells are major effector cells in allergic airway inflammation (13, 14). Recruitment of eosinophils into the inflamed lung tissue is dependent on the interaction of vascular adhesion molecules (VCAM-1, ICAM-1 and selectins) with ligands of the eosinophil surface such as α_4 -integrin (14). The expression of adhesion molecules in the vascular endothelial cells is regulated by transcription factors such as NF- κ B (17). The increased expression of Pur α in the endothelial cells of the inflamed lung may induce the expression of cell adhesion molecules. We found eosinophils contain Pur α , but it is unclear whether the increased Pur α in the inflamed lungs is due to the enhanced expression in each eosinophil or the increased number of eosinophils. Examination of Pur α expression in the circulating blood eosinophils and bone marrow eosinophils also must be examined. Furthermore, cDNA cloning and specific antibody for 45-kDa Pur α are inevitably necessary for evaluating the physiological functions of 45-kDa Pur α in rat lung and eosinophil.

In summary, rat lung and eosinophils contain a larger 45-kDa Pur α that is markedly increased with allergic airway inflammation, although the pathological significance for this increase is unclear at present.

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