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Lung ''Surfactant Convertase'' Is a Member of the Carboxylesterase Family

S. Krishnasamy,*,† N. J. Gross,*,†,‡,[1] A. L. Teng,† R. M. Schultz,* and R. Dhand†,‡

**Department of Molecular and Cellular Biochemistry, Stritch School of Medicine, Loyola University of Chicago, Maywood, Illinois 60153; and †Research Service and ‡Department of Medicine, Hines VA Hospital, Hines Illinois 60141*

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The extracellular conversion of lung surfactant from tubular myelin to the small vesicular form has previously been shown to require a serine-active enzyme called "surfactant convertase." In the present study, a 72kD serine-active enzyme previously identified in mouse lung alveolar lavage and having convertase activity was partially sequenced. Sixty-eight residues obtained from amino acid sequencing of this protein show that it is a new member of the mouse carboxylesterase family (EC 3.1.1.1). The 72kD lung protein also has esterase activity. A commercial esterase of the same family was able to reproduce surfactant convertase bioactivity *in vitro,* unlike several serine proteinases previously tested. We conclude that surfactant convertase is a carboxylesterase which mediates a biochemical step in the extracellular metabolism of surfactant. © 1997 Academic Press

Lung surfactant, the lipoprotein complex that maintains alveolar stability, exists in several structural subtypes that are in sequential relation to each other, namely lamellar bodies (LB), tubular myelin (TM), a surface film, and a small vesicular form (SV)(1). Some of these transformations, e.g. LB to TM, proceed spontaneously in the alveolar environment, but the conversion of TM to SV requires the action of a serine-active enzyme on the basis of its inhibitability by diisopropylfluorophosphate (DFP) (2). We identified a DFP-binding protein of Mr 72kD in mouse alveolar lavage that, in partially purified form, was able to mediate the conversion of TM to SV *in vitro* and called it "surfactant convertase" (3). Initially thought to be a serine proteinase, the activity of this enzyme was found to be inhibitable in part by some lipase inhibitors (4).

We report here the further purification of the 72kD protein from mouse lung lavage. Its partial amino-acid sequence shows it to be a new member of the carboxylesterase family (EC 3.1.1.1) whose members employ the serine catalytic triad (5). When partially purified in its active state this protein has both esterase activity and the ability to mediate the conversion of surfactant subtypes *in vitro.* A closely related carboxylesterase from pig liver was also able to mediate the conversion of surfactant subtypes *in vitro,* unlike any of a range of typical serine-active proteinases. The 72kD DFP-binding protein from mouse lungs therefore appears to be a carboxylesterase and identical to "surfactant convertase" whose role in mediating surfactant subtype conversion we previously described (2,3).

MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO), and solvents were obtained from Fisher Scientific (Itasca, IL) unless otherwise stated. All experiments were performed on female CF#1 mice (Charles River Labs, Wilmington MA) 15-20 weeks old. Mice were killed by an intraperitoneal injection of 10mg pentobarbital and transection of the abdominal aorta. For "cycling experiments" (below) mice were injected 15h prior to killing with 5μCi [^{3}H]-choline (81Ci/mM, Dupont NEN, Boston, MA).

Purification of DFP-binding protein from alveolar lavage. Lungs were lavaged through the catheterized trachea using alveolar lavage (AF) buffer (5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 150 mM NaCl and 2 mM $CaCl_2$). Lavages from groups of 10-20 mice were pooled and centrifuged at 1,000xg for 5 min to remove macrophages and cellular debris. The supernatant was incubated with [^{3}H]-DFP (0.1μCi/ml, Dupont NEN, Boston, MA) for 30 min in the cold, following which it was incubated with Concanavalin-A (Con-A) immobilized on sepharose beads (Pharmacia Biotech, Piscataway, NJ, 1:20, vol:vol) for 30 min. The beads were sedimented by centrifugation, washed three times with AF buffer, and eluted with 20 mM methyl α-D-mannoside in AF buffer. The Con-A purified proteins were precipitated by addition of 2 volumes of ice-cold acetone, resuspended in a small volume of AF buffer and subjected to preparative native (non-denaturing) gel electrophoresis in the cold on a 7.5% gel (6) without a stacking portion. The location of the single [^{3}H]-DFP-binding protein band was identified by autoradiography in preliminary experiments; thereafter, the portion of the gel corresponding to its position was cut from the gel and electro-eluted (Pharmacia Gel Eluter). The gel-eluted band was used for all subsequent experiments described in this paper, including SDS-PAGE and amino-acid sequencing. For surfactant con-

[1] To whom correspondence should be addressed. Fax: (708) 216-2319.

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version bio-assays, the protein was purified as described above from lavage that was not radiolabeled.

Amino-acid sequencing. The native gel-eluted proteins were further separated by 10% SDS-PAGE as described by Laemmli (7), stained with coomassie blue and subjected to autoradiography, revealing a single radiolabeled band, Mr 72kD, plus 2 minor unlabeled proteins. The proteins were blotted to a PVDF immobilon membrane, the band corresponding to the 72kD DFP-binding protein was cut and subjected to micro-sequencing (Worcester Foundation for BioMedical Research, Shrewsbury, MA). The protein was digested with trypsin and the peptides were purified by reversed-phase HPLC. The N-terminus and 3 internal peptides were sequenced.

Bioassay of convertase activity in vitro. Mouse lung lavage was prepared as detailed above from mice that had received [^{3}H]-choline. After initial low speed centrifugation, unlabeled DFP, final concentration 10mM, was added to the surfactant-containing supernatant and incubated on ice for 30min to inhibit endogenous convertase activity. The large aggregate TM surfactant subtype was sedimented by centrifugation at 25,000 rpm for 2h in a Beckman SW28 rotor and resuspended in a small volume of AF buffer and used directly as substrate for surfactant subtype conversion assays (8).

The principle of the *in vitro* assay is that TM is converted to SV by cyclic expansion and compression of the surface area of the surface of a surfactant suspension ("cycling") only in the presence of convertase (2). The extent of conversion is determined by centrifuging the assay mix to equilibrium in a continuous sucrose gradient from which the amounts of the various subtypes can be quantitated by their characteristic buoyant densities (9). Assays were carried out as previously described (8) on aliquots of 100μg of [^{3}H]-choline labeled surfactant in the presence or absence of the 72kD protein as prepared above in a total volume of 2 ml. After 2h cycling the material was underlayed with a continuous (0.9 to 0.1 M) sucrose gradient in AF buffer and centrifuged at 45,000rpm (185,000xg) in a SW55 rotor for 18 h. Fractions of 0.2 ml were dripped out from the bottom and their refractive index and radioactivity were determined. Surfactant subtypes were identified from the peaks in the radioactivity profiles by their characteristic buoyant densities (9) and quantitated by integration of areas under the peaks with the use of commercial software (Peakfit, Jandel Scientific, San Rafael, CA).

Carboxylesterase activity staining. This was performed on 7.5% native gels in the cold. Following electrophoresis the gel was soaked in 100 mM phosphate buffer (pH 6.5) for 1h, and then soaked in fresh phosphate buffer containing 5mM 1-naphthylacetate and 0.4mg/ml Fast Red TR. The formation of a red/brown insoluble complex indicated the presence of esterase activity.

Other methods. Protein concentration was determined with Bio-Rad protein assay kit using albumin as standard. Porcine liver carboxylesterase (Sigma) was further purified by native gel electrophoresis (6). The location of esterase activity was determined on preliminary gels and excised and electroeluted from subsequent gels to obtain a band that was pure on SDS-PAGE.

RESULTS

Purification of 72kD DFP-binding protein. As convertase bioactivity had previously been shown to be inhibited by DFP (2,3), we reacted starting material with [^{3}H]-DFP to aid in tracking the protein of interest. Alveolar lavage fluid that was so treated was subjected to Con-A chromatography which enriched the 72kD DFP-binding protein about 10-fold, fig 1. Further purification was achieved through native gel electrophoresis. The [^{3}H]-DFP-binding band on the native gel was electroeluted and subjected to SDS-PAGE. This yielded a 72kD [^{3}H]-DFP-binding band as the predominant protein with 2 other minor bands each containing lesser amounts of protein, fig 1(lane 5). When electroeluted, the 72kD DFP-binding band contained a single protein as determined by amino-acid sequencing (below). The Mr of this protein was determined by mass spectrometry (time-of-flight analysis) and found to be 66.9kD, in acceptable agreement with SDS-PAGE.

Amino-acid sequences. Four partial amino-acid sequences were obtained, as shown. The amino-acid residues of the 72kD DFP-binding protein are numbered with reference to a previously sequenced mouse liver carboxylesterase (5), differences are shown in bold above the corresponding residues in that enzyme:

19-his ser leu leu pro pro val val asp thr thr gln gly lys val-33

447-arg pro gln **met** val glu gly asp his gly asp glu ile phe **ser** val phe gly ala pro leu leu lys-469
thr phe

511-glu gly tyr leu gln ile gly ala thr thr gln gln ala gln arg-525

526-leu lys ala glu glu val ala phe trp thr glu leu **pro** ala lys-540.
leu

Two substitutions occur in the peptide fragment sequence that aligns with residues 447-469, which contains the histidine residue associated with the serine-catalytic triad. The other substitution occurs in the peptide that aligns with a region close to the carboxyl terminus, residues 526-540. No substitutions were found in the first 15 amino-acids of the fragment corresponding to the N-terminus.

The DFP-binding band of the native gel that was electroeluted to obtain enzyme for convertase assays also contained 2 other protein bands when further analyzed on SDS-PAGE, fig 1. These contaminant bands were separately submitted for amino-acid sequencing and were found to correspond to isoforms of mouse alpha-1 antitrypsin.

Convertase activity. Because the 72kD protein was denatured by purification to identity, it was only possible to perform bioassays for convertase activity on the protein as purified through and electroeluted from native gels. The material purified through native gel

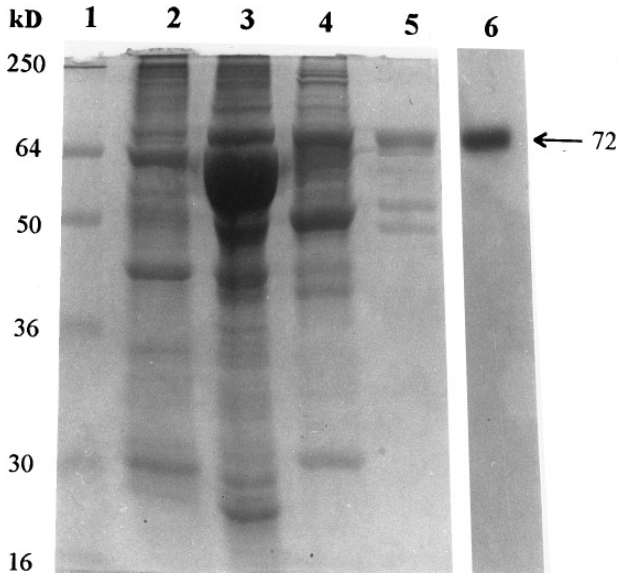

FIG. 1. SDS-PAGE and autoradiogram of lung proteins. Lane 1, protein Mr standards. Lanes 2-5, coomassie blue stain; lane 2, total lung proteins; lane 3, total alveolar lavage proteins; lane 4, Con-A purified proteins; lane 5, native gel purified proteins. Lane 6, autoradiogram of native gel purified [^{3}H]-DFP labeled alveolar lavage proteins.

stage still contains several proteins, fig 1, however the 72kD protein is both the predominant and the only DFP-binding protein in SDS-PAGE. The ability of the Con-A purified protein and the 72kD protein (as eluted from native gels) to mediate surfactant conversion from TM to SV forms were assayed (three experiments). The results were consistent and showed that subtype conversion was negligible in the absence of enzyme or the enzyme treated with 10mM DFP, but proceeded when the enzyme was present. An example is shown in fig 2 (lanes 5 and 6).

Esterase activity. Because the amino-acid sequence of the 72kD DFP-binding protein indicated considerable homology with previously described carboxylesterases, the esterase activity of the 72kD protein was assayed. This was performed in the native gels (Methods) and showed strong esterase activity by the DFP-binding band, fig 3 (lane 1). Esterase activity was completely inhibited by prior treatment of the protein with 10mM unlabeled DFP (fig 3, lane 2). Also shown in fig 3 is an autoradiograph of [^{3}H]-DFP labeled alveolar lavage purified protein and electrophoresed on the same native gel, showing that the location of esterase activity aligns with the DFP-binding.

Convertase activity of porcine liver esterase. We assayed the ability of a commercial esterase of the EC 3.1.1.1 family to mediate surfactant subtype conversion *in vitro*. As this enzyme, as commercially obtained, contains many protein bands on SDS-PAGE, it was first purified on native gels. The (single) band that displayed esterase activity was electroeluted and assayed for convertase biological activity. The results, Table 1, show that liver esterase was able to promote surfactant subtype conversion in dose-response manner, an action that was also inhibitable with DFP.

DISCUSSION

Previous reports from this laboratory, using the *in vitro* "cycling" method described above, suggested that a serine-active enzyme was required to promote the extracellular conversion of TM surfactant to the SV form (2,3). A search for a candidate enzyme in alveolar lavage revealed a single DFP-binding protein of Mr72kD (3). In partially purified form, this protein was able to promote the conversion of TM to SV *in vitro* (3,10). The present studies were performed to identify the nature of the 72kD protein and to further clarify whether this protein, and related carboxylesterases, had the ability to mediate surfactant subtype conversion.

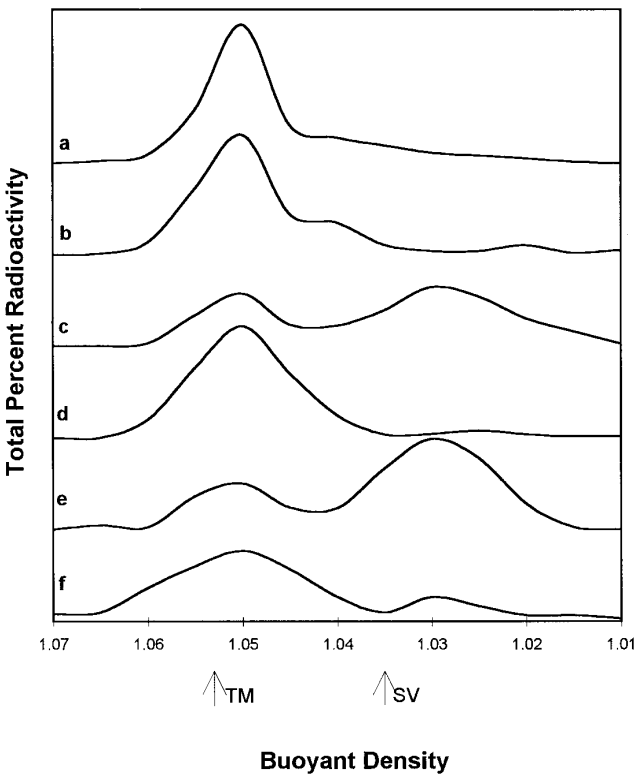

FIG. 2. Cycling assays of convertase activity of various preparations. Each curve represents the profile of 100 μg [^{3}H]-labeled surfactant following sucrose gradient separation before, gradient a, or after cycling with various treatments, gradients b-f. Arrows along abscissa represent the characteristic buoyant density peaks of TM subtype (1.050gm/ml) and SV subtype (1.030gm/ml). Gradient b, cycled without additives; c, cycled with Con-A purified proteins (35μg); d, cycled with Con-A purified proteins plus 10mM DFP; e, cycled with native gel-purified protein (24μg); f, cycled with native gel-purified protein plus 10mM DFP.

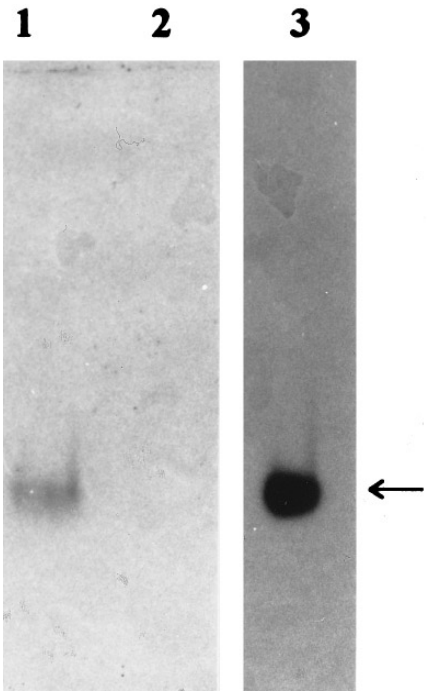

FIG. 3. Esterase activity and [^{3}H]-DFP autoradiogram on native gels. Lavage fluid proteins were preincubated with [^{3}H]-DFP and purified through Con-A chromatography and native gel electrophoresis. Separate lanes of the same gel were stained for esterase activity (lane 1: native gel purified protein; lane 2: native gel purified protein preincubated with 10mM DFP), or subjected to autoradiography (lane 3: [^{3}H]-DFP labeled protein), showing that esterase activity is resolved to the same position as [^{3}H]-DFP binding.

The results above provide strong evidence that the 72kD DFP-binding protein that was previously identified on SDS-PAGE of lung lavage is responsible for surfactant convertase activity. This evidence is not conclusive because it has not been possible to purify this protein to identity in an active state. However, it is clear that convertase bioactivity is inhibited by DFP (2) and only one DFP-binding protein was identified on native gel electrophoresis, and this was the predominant protein in the gels. Moreover, the two contaminating proteins were found, on amino-acid sequencing, to represent isoforms of mouse alpha-1 antitrypsin (a serine proteinase inhibitor) and not likely, therefore, to be capable of mediating serine-active enzyme bioactivity. It seems reasonable to conclude that the 72kD DFP-binding protein is, in fact, identical with "surfactant convertase" (2).

Partial amino-acid sequences of this protein in pure form show that it is a new member of the carboxylesterase family. Of the 68 residues obtained by sequencing, 3 differences were found from a previously published mouse carboxylesterase (5). These variations do not represent isoforms, organ or strain differences from the previously published protein because our preliminary sequence analysis of partial cDNA clones obtained from a mouse lung library indicate the presence of transcripts that correspond to both the novel amino-acid sequence we report above as well as the previously published mouse liver carboxylesterase sequence (preliminary data, not shown).

Other lung carboxylesterases have been described, including from rat (11) and human lungs (12), however their homology is very poor with mouse "surfactant convertase."

It had previously been thought that surfactant convertase was a serine proteinase on the basis of its inhibition by DFP and, to some extent, by a range of other serpins but not by inhibitors of other classes of proteinases (2). It had not been previously possible to elicit any proteinase activity using a wide range of synthetic proteinase substrates (data not shown). Nor were we able to reproduce the biologic activity of convertase *in vitro* by a range of typical serine proteinases, including urokinase, elastase, trypsin, and alpha-chymotrypsin (10). There was some evidence that convertase had lipase properties (4), and that phospholipase D (but not other phospholipases) could reproduce convertase activity *in vitro* (AL Teng, unpublished). The present finding that convertase has sequence homology with the carboxylesterase family was therefore unexpected, and confirmation that it has carboxylesterase action was sought. The partially purified protein clearly has esterase activity that is DFP-inhibitable, fig 3. We therefore tested the possibility that other carboxylesterases could mediate surfactant conversion *in vitro* using purified porcine liver esterase, a serine-esterase. We found, Table 1, that this enzyme was able to reproduce the action of surfactant convertase on surfactant in dose-response fashion, -an action that was DFP inhibitable. This finding supports the notion that surfactant convertase is a carboxylesterase.

The present results indicate that surfactant con-

TABLE 1

Activity of Porcine Liver Carboxylesterase in Cycling Assay

Reaction with substrate	Reaction products, % of total	
	TM%	SV%
Uncycled without enzyme	91.5	8.5
Cycled without enzyme	89.9	10.1
Cycled with 24 μg porcine esterase	33.2	66.8
Cycled with 48 μg porcine esterase	22.5	77.5
Cycled with 72 μg porcine esterase	20.5	79.5
Cycled wtih 72 μg porcine esterase plus 10 mM DFP	89.2	10.8

Note. Assays consisted of 100 μg [^{3}H]-choline labeled TM surfactant, cycled or not cycled for 2h with the indicated enzyme and inhibitor amounts. The products were separated on sucrose gradients (Materials and Methods) and identified and quantitated by buoyant density.

vertase is a novel member of the carboxylesterase family. Its substrate remains unclear, -carboxylesterases are known to have wide tissue distribution and to have a wide range of substrate specificities (13). The molecular action of convertase on TM surfactant, which undergoes major structural and biochemical transformations when it is converted to the SV form, remains to be elucidated. However, the facts that it is a member of the carboxylesterase family, has esterase activity, and that esterase activity is sufficient to mediate the conversion of TM to SV provide direction for further studies.

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