

REVIEW

The role of lipocalins in airway disease

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Summary

The pathogenesis of allergic airway inflammation and disease is complex and still not fully understood. Many cells, factors and mediators are involved in the different aspects of induction, maintenance and persistence of airway inflammation. The heterogeneity and redundancy of this system is one of the main reasons why novel therapeutic targets focusing on the pathogenesis of asthma only hesitantly reach the market and clinical application. Thus, it seems mandatory that we proceed in our efforts to better understand this micro cosmos to succeed in the development of safe and effective drugs for the treatment of more severe and refractory forms of asthma and chronic obstructive pulmonary disease. One of the more recently discovered mediators in the context of airway inflammation are the lipocalins (Lcns). They are a family of proteins that share functional and structural similarities and are involved in the transport of small hydrophobic molecules such as steroids and lipids into the cell. Lcns are found in many different cell types from plants and bacteria through invertebrate cells to cells of vertebrate origin. The purpose of this review is to summarize the role of Lcns in airway diseases, focusing on allergic and infectious inflammation. In particular, we will summarize the present knowledge about Lipocalin 1 and Lipocalin 2, where exciting new discoveries in the recent years have highlighted their role in pulmonary disease and infection. This new class of proteins is another putative candidate for the development of novel drugs against airway inflammation.

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Introduction

Lipocalins (Lcns) are a family of proteins that share functional and structural similarities which are a) the transport of small hydrophobic molecules such as steroids, bilins, retinoids, and lipids into the cell and b) regions of sequence homology and a common tertiary architecture [1–5]. Overall, the sequence conservation between different members of the lipocalin protein family is low, and alignment is only possible within particular region sequences. Albeit this low grade of sequence homology, we have created a 'pseudo-phylogenetic' tree of kernel and outlier lipocalins which may serve as a graphic reference to the major members of the lipocalin family and their sequence relationship. The 'kernel' lipocalins are characterized by three common conserved stretches of residues, while 'related proteins' to lipocalins match no more than two of these three diagnostic motifs (Fig. 1). Furthermore, according to sequence alignments, we subdivided the 'kernel' lipocalins into three subgroups, namely in lobe 1: prostaglandin D synthase (PGDHs); neutrophil lipocalin (NGALs) and K-1-microglobulin (α 1Ms); in lobe 2: retinol-binding proteins

(RBPs); bilin-binding proteins and apolipoprotein D (apoD); and in lobe 3: mouse major urinary proteins (MUPs) and γ -lactoglobulins (β -lg).

The common tertiary structure is much more consistent between the different members of the family than the sequence homology. It is comprised of an eight-stranded, anti-parallel symmetric beta-barrel core forming a calyx with a binding site for a small molecule [3, 4]. Developmentally preserved, these proteins are found in many different cell types from plants and bacteria through invertebrate cells to cells of vertebrate origin.

Given the heterogeneity of a family defined by structure, Lcns have been associated with many biological processes, among them pheromone transport, retinoid binding and cancer cell interactions. Various reports have suggested a role for Lcns in the development of airway diseases, such as the interaction with innate immune responses and in particular with inflammation and detoxification processes, prostaglandin synthesis and cell growth and repair. The role of Lcns in airway diseases, excluding neoplastic processes (summarized in Table 1), will be the focus of this review.

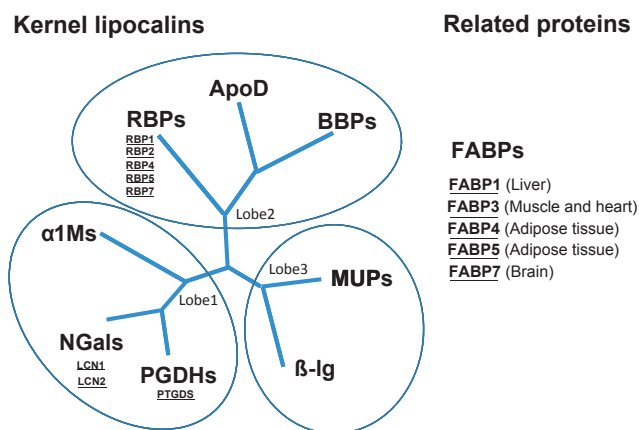


Fig. 1. An unrooted pseudo-phylogenetic tree of kernel lipocalins and selected outlier lipocalins. Overall, the sequence conservation between different members of the lipocalin protein family is very low. Sequence alignment is only practicable within particular regions. The 'kernel set' of lipocalin sequences is characterized by three short conserved stretches of residues. [4, 82]. Human lipocalins which have been associated with pulmonary disease and infection are marked by underlined gene symbols. Fatty-acid-binding proteins (FABPs), although they have ten beta-strands in the barrel and therefore are not strictly lipocalins, were included in the figure due to structural resemblance and relation to pulmonary diseases (adapted from [82]).

In particular, we will focus on Lipocalin 1 (Lcn1) and Lipocalin 2 (Lcn2), where exciting new discoveries in the recent years have highlighted their role in pulmonary disease and infection. Then, we will discuss the role of prostaglandin D synthase in the context of its eminent role in prostaglandin synthesis. Finally, we will provide an overview on the role of the fatty-acid-binding proteins and the retinol-binding proteins that belong to the lipocalin family and their role in pulmonary diseases.

Lcn1

Lipocalin 1 is also known as tear prealbumin due to its initial localization [6] and most prominent expression site. Lcn1 is a major component of tears and consists of several isoforms with a molecular mass between 17 and 18 kDa. Other names for Lcn1 include VEG protein, tear lipocalin, PMFA and Von Ebner gland protein.

Initially believed to be secreted exclusively by the lacrimal glands into the tear fluid, it is now known that Lcn1 is secreted by numerous exocrine glands, such as the lingual glands, sweat glands, prostate and secretory glands of the tracheobronchial tract, as well as the nasal mucosa [7]. However, the role of Lcn1 in the airways has not yet been carefully delineated, and most results discussed below are obtained from *in vitro* studies or from studies on ocular tissues or secretions.

Early studies had suggested an antimicrobial activity of Lcn1, either by an inherent microbiocidal property or via enhancement of lysozyme activity [8]. More

recent studies show that Lcn1, similar to Lcn2, whose contribution to airway disease has been studied more intensively, binds to different bacterial siderophores, among them bacterial catechol-type enterobactin and hydroxamate-type desferrioxamine B, and all major classes of fungal siderophores. Exogenous Lcn1 can inhibit bacterial and fungal growth under iron-limiting conditions, suggesting that Lcn1 might serve as a member of the innate immune system especially involved in mucosal defence against fungal infections [9]. However, the *in vivo* effects of this property have not yet been elicited. Lcn1 has been shown to bind to an unusually high number of hydrophobic ligands, in particular lipids, and to limit the harmful effects of lipid peroxidation products *in vitro* [10]. Early studies also showed that Lcn1 has a cysteine protease inhibitor activity [11].

Recently, more data specifically related to airway diseases have been obtained. The expression of Lcn1, similar to that of Lcn2, is modulated in different pulmonary disease: when comparing patients with chronic obstructive pulmonary disease (COPD) to healthy smokers, Lcn1 was markedly reduced in COPD patients, with its levels correlating to certain parameters of lung function, indicating a relationship to disease severity [12]. The authors suggested that this relationship reflected the increased susceptibility of COPD patients to infectious exacerbations and the potential role of Lcn1 in innate defence mechanisms. In saliva, on the other hand side, Lcn1 was significantly up-regulated in heavy smokers, compared with non-smoking volunteers; however, a comparison to the levels of smokers with COPD was not made [13]. Similar results were obtained when comparing Lcn1 levels in nasal and bronchoalveolar lavage fluids from smokers and non-smokers [14]. Western blot analysis of bronchial secretions of patients with cystic fibrosis also showed enhanced secretion of Lcn1 compared with healthy controls [15].

Taken together, different authors concluded that Lcn1 might serve as a scavenger of potentially harmful, lipophilic substances and thus serve as a general protection factor for epithelia and as a non-specific innate defence mechanism against microorganisms [7].

Lcn2

Lipocalin2 is also named: neutrophil- gelatinase-associated lipocalin, NGAL, oncogene 24p3, SIP24 or uterocalin. Lcn2 is a 25-kDa protein with the above mentioned lipocalin tertiary structure. Right from the start, after describing its expression in neutrophils and cloning its structure, Lcn2 was assigned a putative immunomodulatory role. This was supported by its binding capacities to the bacterial derivative formylpeptide FMLP [16] even though this property was later refuted [17].

Table 1. Lipocalin family members with reported expression locus and respective reference in the text

Family name	Family member	Tissue expression	Other names	Reference
Fatty-Acid-binding proteins	FABP1	Liver, Alveolar Macrophages, Type II Pneumocytes	FABPL; L-FABP	[67]
	FABP2	Intestinal	FABPI; I-FABP; MGC133132	[68]
	FABP3	Muscle and heart, Type II Pneumocytes, Macrophages, Fibroblasts	MDGI; FABP11; H-FABP; O-FABP; M-FABP	
	FABP4	Adipose tissue, BAL, Macrophages, Airway Epithelium	aP2; A-FABP; ALBP AFABP	[70, 71]
	FABP5	Adipose tissue, BAL, Type II Pneumocytes, Macrophages, Fibroblasts	EFABP; E-FABP; PAFABP; PA-FABP; OTTHUMP00000166022	[68, 71]
	FABP6	Intestinal (ileal)	ILBP; I-15P; I-BAP; ILBP3; ILLBP; I-BABP; I-BALB, Gastrotropin;	[73]
	FABP7	Brain, Lung	MRG; BLBP; FABPB; B-FABP; DKFZp547J2313	
Lipocalins	LCN1	Lingual, Sweat, Tear, Upper and Lower Airway Glands, BAL, Sputum	Lipocalin-1, MGC71975, PMFA, Tear lipocalin, Tear prealbumin, Tlc, TLC, TP, VEGP, VEG protein,	[7, 12, 14, 15]
	LCN2	Neutrophils, Macrophages, Dendritic Cells, Trachea, Lung, Sputum, BAL, Stomach, Small Intestine, Pancreas, Kidney, Prostate, Thymus	24p3, 25 kDa alpha-2-microglobulin-related subunit of MMP-9, HNL, Lipocalin-2, MSFI, Neutrophil gelatinase-associated lipocalin, NGAL, Oncogene 24p3, p25	[16–21]
	LCN8	Epididymis	EP17, Epididymal-specific lipocalin-8	[76]
	LCN9	Epididymis	Epididymal-specific lipocalin-9, MUP-like lipocalin	
	LCN10	Epididymis	Epididymal-specific lipocalin-10	
	LCN12	Epididymis	Epididymal-specific lipocalin-12, MGC34753, MGC48935	
Retinol-binding proteins	RBP1		Cellular retinol-binding protein, Cellular retinol-binding protein I, CRABP-I, CRBP, CRBP1, CRBPI, CRBP-I, RBPC, Retinol-binding protein 1	[76]
	RBP2	Lung fibroblasts	Cellular retinol-binding protein II, CRABP-II, CRBP2, CRBP2, CRBP-II, RBPC2, Retinol-binding protein 2	
	RBP4		Plasma retinol-binding protein, PRBP, PRO2222, RBP, Retinol-binding protein 4	
	RBP5		Cellular retinol-binding protein III, CRBP3, CRBP3, CRBP-III, HRBPiso, Retinol-binding protein 5	
	RBP7		Cellular retinoic acid-binding protein 4, Cellular retinoic acid-binding protein IV, CRABP4, CRABP-IV, CRBP4, CRBP4, MGC70641, Retinoid-binding protein 7	
Prostaglandin D synthases	PTGDS	CNS, CFS, Plasma, Urine, Eyes, Male Genitalia, Heart, Mast cells,	Beta-trace protein, Cerebrin-28, Glutathione-independent PGD synthase, Lipocalin-type prostaglandin-D synthase, LPGDS, L-PGDS, PDS, PGD2, PGD2 synthase, PGDS, PGDS2, Prostaglandin-D2 synthase, Prostaglandin-H2 D-isomerase	[49–58]

Further studies suggested that Lcn2 might play a role in antimicrobial defence and the regulation of inflammation and possibly cell growth [17] in the respiratory

tract. Human and mouse Lcn2 are expressed in trachea and lungs under non-inflammatory conditions [18]. Human Lcn2 is up-regulated in lung epithelium through

inflammation, comprising expression in goblet cells and type II pneumocytes [19]. Similarly, Lcn2 is up-regulated in murine macrophages after Lipopolysaccharide (LPS) stimulation [20] and in lung tissues of LPS-treated mice [21].

Lipocalin 2 function remained unclear for many years, partly due to the fact that relevant *in vivo* binding candidates for Lcn2-mediated transport remained unknown. Hence, structural studies aimed to identify those ligands provided first clues to Lcn2's role *in vivo*. Lcn2 binds to enterobactin-type bacterial siderophores, which facilitate iron uptake by bacteria and thus interfere with the siderophore-mediated iron-uptake necessary for growth of different pathogens such as *Escherichia coli* [22]. Subsequent studies revealed that Lcn2 also binds to other types of siderophores, such as carboxy-mycobactin (produced by mycobacteria) and bacillibactin (produced by *Bacillus anthracis*) [23, 24]. In addition, Lcn2 has been proposed to bind to an as yet unknown mammalian siderophore [25, 26]. More insight into Lcn2's role in innate immunity and its ligands, enterochelins and iron-chelating proteins has been gained very elegantly by knock-out (KO) experiments. Mice genetically lacking Lcn2 showed a marked increase in mortality in an *E. coli*-induced sepsis model [27]. The creation of the KO tool allowed unravelling of Lcn2's role in the defence against other pathogens as well and, in particular, in the airways. Thus, consecutive studies showed that Lcn2 has a role in the host defence against mycobacteria, where Lcn2 uptake by epithelial cells inhibited intracellular growth inside the epithelial cells [28]. In the same line, Lcn2 is required for pulmonary host defence against *Klebsiella* infection [29] and is protective against *E. coli* pneumonia [30] and up-regulated by *Chlamydia pneumoniae* infection [31]. Yet, further studies revealed a role for Lcn2 in other airway diseases not initiated by pathogens. In our own hands, Lcn2 was up-regulated in the acute and chronic stages of allergic airway inflammation [32]. These studies revealed a protective effect for Lcn2 against airway inflammation, possibly due to interference with its known pro-apoptotic properties [25]. Furthermore, recent years have shed light on the induction requirements of Lcn2 with possible relevance to airway diseases; It had been known for some time that Lcn2 can be induced by pro-inflammatory cytokines such as IL-1 β [19]. More recent studies demonstrated Lcn2's induction to be TLR4-dependent [33] yet in certain disease models to be Myd88-independent [31] and inducible by TNF- α , IL-4 [32] and IL-17 [34, 35].

However, not only functional studies have suggested a role for Lcn2 in airway diseases, since Lcn2 is constitutively expressed in neutrophilic granules [36]. Thus, increases in neutrophils in any disease state can translate into increase in Lcn2 making it a candidate

biomarker for neutrophil activity in body tissues and fluids such as bronchoalveolar lavage (BAL) [37] and sputum [38].

In this line, Lcn2 has been used as a biomarker to correlate changes in Lcn2 levels to different pulmonary pathologies. For example, smokers compared with non-smokers have higher levels of Lcn2 in the BAL fluid which correlated with emphysematous lesions on high-resolution computed tomography [39]. Lcn2 levels in induced sputum were elevated in COPD and asthmatic patients compared with healthy controls [40], where Lcn is possibly correlated with bronchial hyperresponsiveness [41]. Similarly, in a study of 402 patients with COPD and 229 control subjects, Lcn2 plasma levels correlated well with COPD after adjustment for several confounders such as age, smoking, neutrophil blood counts or CrP. In this study, frequent exacerbations and hypoxaemia lead to even higher Lcn2 levels, while higher COPD stages were correlated with a decrease in Lcn2 levels [42]. In a study of 78 asthmatics, levels of the heterodimer of Lcn2 and MMP-9 closely correlated to disease severity. Additionally, BAL cells from severe asthmatics showed impaired decreases in Lcn2/MMP-9 levels in response to steroids [43]. A comparison of 42 cystic fibrosis patients with 25 normal subjects also showed high correlation of Lcn2 to those patients experiencing acute exacerbations or chronic *Pseudomonas aeruginosa* infection [44]. When Lcn was used to predict the development of bronchopulmonary dysplasia (BPD) in a well-executed study of 32 pre-term neonates, consecutive measurements of Lcn in BAL fluid best separated between pre-term infants with acute respiratory distress syndrome who subsequently developed BPD and babies without further complications [45]. However, serum levels of Lcn2 did not correlate well with improvement in lung function (FEV₁) in COPD patients after steroid treatment of exacerbations [46]. Similarly, in another study, no correlation between plasma Lcn2 levels and the development of BPD in pre-term infants were found, whereas other neutrophil markers showed significant correlation [47].

Thus, the question remains whether Lcn2 can serve as a biomarker in airway pathologies to distinguish between healthy controls and patients or different subtypes of disease states. In the kidney, Lcn2 is already widely used to detect and monitor stages of acute renal failure [48]. Thus, suitable detecting assays are already developed making further trials in respiratory diseases to address this interesting issue readily feasible.

PTGDS

True to its lipocalin affiliation, prostaglandin D2 synthase (PTGDS) binds retinoic acid, biliverdin, bilirubin, gangliosides and amyloid β peptides. However, apart

from its function as a protein, PTGDS has enzymatic activity where it converts prostaglandin H₂ into prostaglandin D₂ (PGD₂), an active prostaglandin component. There are two different types of prostaglandin synthases: H-PGDS and L-PGDS, which is the PGDS exhibiting binding property typical of a lipocalin. L-PGDS is localized in the CNS and secreted into the cerebrospinal fluid [49–52], where the human enzyme was initially described as β -trace, which is a major protein in human cerebrospinal fluid. Additionally, PTGDS's expression has been demonstrated in plasma [53], urine [54], ocular tissues [55], male genital organs [56] and seminal plasma and the heart [57].

PGD₂ is involved in smooth muscle contraction/relaxation via the prostaglandin D₂ receptor DP1 [58]. It is also a potent inhibitor of platelet aggregation and mediates chemotaxis of eosinophils and basophils into the lung via prostaglandin D₂ receptor DP2 [59] in the periphery. PGD₂ exacerbates asthma [58], suggesting that PGD₂ is pro-inflammatory. Other studies also suggested anti-inflammatory properties of PGD₂ in the respiratory tract. Injection of PGD₂ or induction of PGD synthases in the lung was shown to suppress inflammatory processes elicited by bleomycin and urate crystals [60–62]. In addition, PGD₂ in a murine model of pleuritis caused by carrageenan was associated with resolution of lung inflammation. These results suggest that the effects of PGD₂ production depend on the inflammatory milieu. Taken together, PTGDS via synthesis of PGD₂ are certainly an interesting target for closer investigation of disease development in the respiratory tract.

Some suggestions for PTGDS' role in airway disease were obtained by studies of transgenic human PTGDS-overexpressing mice (TG) in a model of ovalbumin/alum induced airway inflammation (AI). Although allergic sensitization seemed unaffected by overexpression of PTGDS, WT and TG mice differed significantly after allergen airway challenges; inflammatory cells in BAL were significantly enhanced in TG mice with significant increases in eosinophils and lymphocytes, most likely due to increased levels of IL-4, IL-5 and eotaxin, and a decrease in IFN- γ production [63]. A more recent study showed results in the same line using a selective inhibitor of PTGDS in a similar model [64]. However, neither of these studies looked into *in vivo* effects of endogenous PTGDS on airway disease. Joo et al. [65] examined this question in more detail. They showed that expression of PTGDS in macrophages was not limited to those macrophages infiltrating atherosclerotic plaques [66], but could also be induced by LPS or *Pseudomonas* infection in whole lung and alveolar macrophages. Improved clearance of *Pseudomonas* in the PTGDS TG mice or after intratracheal instillation of PGD₂ to WT mice further suggested that PTGDS might

be yet another lipocalin with interesting anti-inflammatory or antimicrobial activity in the respiratory tract.

Fatty-acid-binding proteins

The fatty-acid-binding proteins, FABP1–7, share a lipocalin structure and hence transport capacity for long chain fatty acids, retinoids and eicosanoids. They have been implicated in the transport of fatty acids in general, the transfer of fatty acids between extra- and intracellular membranes and also in the modulation of cell growth and proliferation. They are divided into three subtypes by expression locus, namely the hepatic, the intestinal and the heart subtype, with different expression loci found for the different members of the family (Table 1).

Few of the FABP have been investigated in direct context with the respiratory tract or pulmonary diseases. However, a limited number of studies associated these molecules with airway diseases and are discussed in the following.

FABP1 correlated with the degree of lung injury in a model of acute respiratory failure, in line with its increased expression in alveolar macrophages and type II pneumocytes [67].

FABP3 and FABP5 are both expressed in rat lungs in alveolar epithelial type II cells, fibroblasts and macrophages [68] and are involved in surfactant synthesis. Double KO mice of FABP3 and FABP5 exhibited a defect in surfactant expression severely disrupting the breathing physiology [69].

FABP4, initially described in adipocytes and macrophages, is also expressed in human airway epithelial cells, where it is up-regulated via IL-4 and -13. Studies in a classic OVA/Alum i.p. model of AI demonstrated that allergic AI was dependent on epithelial FABP4 expression, as KO mice and bone marrow chimeras, selectively expressing FABP4 in structural cells, showed significantly decreased AI compared with WT animals [70]. Whether these effects were achieved via binding of eicosanoids, such as LTA₄, key players in allergic airway disease, thus inhibiting their degradation remains to be shown. FABP4 levels are elevated in non-human primate models of bronchopulmonary dysplasia and also in human samples [71].

FABP5 is expressed by alveolar macrophages and alveolar epithelial cells type II and its levels are increased during acute rejection in a rat model of orthotopic lung transplantation [72].

FABP7 is underexpressed in congenital lung malformations [73].

With these preliminary studies suggesting disparate roles for the different FBP in different airway diseases, substantial data addressing their function in these particular diseases are still lacking and warrant future research in this area.

Retinol-binding proteins

Vitamin A or retinoic acid has been discussed for a long time as being a crucial factor in lung development [74,75]. Lung repair processes with the development of new alveoli in adult lungs may also be regulated by retinol and retinol-binding proteins (RBPs) [76] making them an attractive target for therapy in diseases where alveolar surface area is lost, such as bronchopulmonary dysplasia, emphysema and COPD, and more broadly and general in aging where this process also occurs. In this line, retinoic acid reduces lung septation failure during lung development [77], and different types of emphysema [78, 79]. Clinical trials to assess the benefits of Vitamin A supplementation in very low birth weight (VLBW) infants showed a modest protective effect on the development of bronchopulmonary dysplasia [80], suggesting that retinol and therefore the inversely regulated retinol-binding proteins do, indeed, play a crucial role in lung development and physiology. Plasma RBP levels are increased in acute exacerbations of cystic fibrosis patients [81] suggesting an additional role for these Lcn family members in the regulation of inflammation, as has been proposed for many other members of the family.

Studies dissecting the role of individual RBPs in respiratory disease are lacking, though. Thus, further investigations are needed to increase our understanding of these members of the Lcn family and their role in airway diseases.

Conclusion

The family of Lcns is large, comprising at least 32 genes in the human genome (Table 1). We have attempted to give an overview of the status quo of

three individual members of this family, Lcn1, Lcn2 and PTGDS and two subgroups, the fatty-acid-binding proteins and the retinol-binding proteins with regards to their role in airway diseases.

A picture is starting to emerge that these proteins play an important role in diverse airway diseases due to diverse functional roles: Especially Lcn1 and Lcn2, but also other family members, play an important role in innate antimicrobial defence mechanisms. Their levels within respiratory tissues correlate with different pulmonary disease states making them a putative biomarker for different pulmonary diseases, similar to their use in acute renal failure where their biomarker potential is already well accepted. Additionally, some of these family members, such as PTGDS and FABs, regulate growth processes also within the lung, suggesting targets for therapeutic interventions, as well. The riddle is yet not fully solved and conflicting data concerning the role of Lcns in airway diseases also exists, in particular for results pertaining to Lcn levels in clinical samples. Additionally, many areas are lacking substantial studies with valid functional models or interventional approaches to put initial observations on a more solid ground. Carefully designed clinical trials with well-characterized study samples will help to evaluate the use of Lcns as biomarkers for different disease entities and their subgroups. It is intriguing to speculate that the future research in this particular field will allow us to formulate novel hypotheses for the development and, even better, for treatment or prevention of airway diseases.

Conflict of interests

The authors declare no conflict of interests.

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