

# Cell-penetrating peptides and proteins: new inhibitors of allergic airways disease<sup>1</sup>

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**Abstract:** Cell-penetrating peptides (CPPs) or protein transduction domains (PTDs) are peptides that have the ability to efficiently traverse cellular membranes, either alone or in association with molecular cargo. Several naturally occurring PTDs, including those from HIV TAT and *Drosophila* antennapedia, have this unique activity. Synthetic CPPs, such as polyarginine, also have the ability to enter cells and transport a variety of cargo. While the precise mechanism(s) of cellular entry for individual CPPs may vary, it is likely that uptake is mediated, at least in part, through endocytosis. Moreover, biological activity of cell-penetrating peptides and proteins has been clearly demonstrated in a number of in vitro and in vivo studies. Recently, cell-penetrating proteins targeting the Ras GTPase and the phospholipid kinase PI3K (phosphoinositide 3-kinase) have been shown to inhibit eosinophil trafficking and survival in vitro. These proteins, as well as CPPs targeting the STAT-6 transcription factor or the T-cell costimulatory molecule CTLA-4 (cytotoxic T lymphocyte-associated antigen-4), have also been tested in animal models of asthma. Data from several groups, including ours, indicate that these molecules inhibit airway eosinophilic inflammation, airway hyperresponsiveness (AHR), and mucus production in experimental allergic airways disease. Thus, CPPs targeting these and other signaling molecules may also effectively inhibit allergic airways disease in humans.

**Key words:** asthma, cell-penetrating peptide, cytokines, CD4<sup>+</sup> T cell, eosinophil, inflammation.

**Résumé :** Les peptides de pénétration cellulaire (PPC) ou les domaines de transduction protéiques (DTP) sont des peptides capable de traverser efficacement les membranes cellulaires, soit individuellement, soit en association avec un cargo moléculaire. Plusieurs DTP naturels, y compris ceux de la protéine TAT du VIH et de la protéine antennapedia de la drosophile, ont cette propriété particulière. Les PPC synthétiques, comme la poly-arginine, ont aussi la capacité d'entrer dans les cellules et de transporter des cargos de structures diverses. Le ou les mécanismes d'entrée des PPC individuels dans les cellules peuvent varier; toutefois, il est probable que la capture s'effectue, du moins en partie, par endocytose. De plus, plusieurs études in vitro et in vivo ont clairement démontré l'activité biologique des protéines et peptides de pénétration cellulaire. Récemment, on a montré que les peptides de pénétration cellulaire ciblant la Ras GTPase et la phospholipide kinase, phosphoinositide 3-kinase (PI3K), inhibent le transport et la survie des éosinophiles in vitro. Ces protéines, ainsi que les PPC ciblant le facteur de transcription STAT-6 ou la molécule de costimulation des lymphocytes T CTLA4 (antigène 4 des lymphocytes cytotoxiques), ont aussi été examinées dans des modèles animaux de l'asthme. Les résultats obtenus chez plusieurs groupes, dont les nôtres, indiquent que ces molécules inhibent l'inflammation éosinophile des voies aériennes, l'hyperactivité bronchique (HRB) et la production de mucus dans la maladie bronchique allergique expérimentale. Ainsi, les PPC ciblant ces molécules et d'autres molécules de signalisation pourraient aussi inhiber efficacement la maladie bronchique allergique chez les humains.

**Mots-clés :** asthme, peptide de pénétration cellulaire, cytokines, cellule CD4<sup>+</sup>, éosinophile, inflammation.

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## Introduction

Asthma is a complex inflammatory disease characterized

by airway eosinophilic inflammatory responses mediated by T helper type 2 (Th2) cells. Asthma is accompanied by mucus hypersecretion, microvascular leakage, airway hyperresponsiveness (AHR), and structural changes to the airways, termed airway remodeling (Wills-Karp 1999). A number of intracellular signaling proteins are implicated in various aspects of the inflammatory processes that regulate asthma pathogenesis, and these proteins are thus targets for therapeutic intervention. Pharmacological inhibitors, which are available for a subset of these molecules, have shown promise in diminishing inflammation and pathogenesis in rodent models of experimental allergic airways disease (Escott et

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al. 2000; Duan et al. 2004; Ziegelbauer et al. 2005). For some signaling molecules, however, particularly those that lack enzymatic activity, pharmacological inhibitors may not be available. Recently, several groups have developed cell-penetrating proteins or peptides targeting specific signaling molecules implicated in allergic airways disease. Each of these molecules contains a short peptide sequence, called a protein transduction domain (PTD) or cell-penetrating peptide (CPP), to promote intracellular uptake of the associated cargo (Lindgren et al. 2000; Schwarze et al. 2000). Abundant data demonstrate that CPPs act as efficient molecular transporters for the delivery of a wide range of molecular cargo, including full-length proteins, peptides, oligonucleotides, and small molecules, across cellular membranes, both in vitro and in vivo (Derossi et al. 1998; Schwarze et al. 1999; Lindgren et al. 2000). We and others have used this technology to investigate the contribution of signaling molecules to inflammation and AHR in murine models of allergic airways disease (Myou et al. 2003a, 2003b; Choi et al. 2006; McCusker et al. 2007). In this review, we summarize the current literature describing the use of cell-penetrating peptides and proteins that target signaling molecules implicated in allergic airways disease.

### Cell-penetrating peptides and mechanisms of cellular entry

To date, a number of naturally occurring CPPs have been described, including those from the TAT transcription factor from human immunodeficiency virus 1 (HIV-TAT) (Frankel and Pabo 1988; Green and Loewenstein 1988), the *Drosophila melanogaster* antennapedia protein (Derossi et al. 1994; Schutze-Redelmeier et al. 1996), and the herpes simplex virus structural protein VP22 (Elliott and O'Hare 1997). Each of these naturally occurring CPPs contains several highly basic amino acids, including lysine and arginine. A number of synthetic CPPs, including PTD4 (a derivative of the HIV-TAT PTD) (Ho et al. 2001), penetratin (a derivative of antennapedia) (Derossi et al. 1996), polylysine (Shen and Ryser 1978), and polyarginine (Mitchell et al. 2000; Futaki 2002; Fuchs and Raines 2004), have also been utilized as molecular transporters for biologically active peptides and proteins. In addition, peptides from human transcription factors, including Hph (Choi et al. 2006) and fibroblast growth factor (Fujihara et al. 2000), contain PTDs.

Several mechanisms have been proposed to explain how PTDs, particularly TAT, antennapedia, and polyarginine, traverse cellular membranes. Here we will provide a brief overview of uptake mechanisms. For further information, several excellent in-depth reviews describing mechanisms of uptake have been published recently (Fischer et al. 2005; Gump and Dowdy 2007; Patel et al. 2007). Some reports suggest that CPPs may be internalized by energy-independent plasma membrane translocation without the formation of membrane vesicles (Zaro and Shen 2005; Zaro et al. 2006; Tunnemann et al. 2006). The majority of reports, however, suggest that CPPs enter cells by using endocytic pathways. The entry of the HIV-TAT PTD, for example, is thought to be energy dependent, involving endocytosis, possibly mediated by clathrin (Richard et al. 2005) or caveolae (Fittipaldi et al. 2003). Lipid raft-dependent macropinocytosis, an actin-driven mechanism in which the cell engulfs

substantial amounts of extracellular fluid into macropinosomes (Wadia et al. 2004; Kaplan et al. 2005), has also been proposed as a mechanism of uptake for TAT and other PTDs. Cellular internalization of synthetic polyarginine may require heparan sulfate (HS) (Fuchs and Raines 2004) or other membrane-associated proteoglycans, which may bind polyarginine-containing molecules during induction of macropinocytosis (Swanson and Watts 1995; Nakase et al. 2004, 2007). Interestingly, after inhibition of macropinocytosis, cellular uptake of polyarginine still occurs, providing evidence that several pathways can regulate uptake (Nakase et al. 2004). Cellular uptake of CPPs is also sensitive to temperature and peptide concentration (Richard et al. 2003; Fretz et al. 2007). Small increases in peptide concentration may significantly enhance peptide localization in the cytosol. While delivery of small amounts of polyarginine results in the appearance of peptide in intracellular vesicles (consistent with uptake via endocytosis), higher concentrations may produce more diffuse, cytoplasmic staining. Uptake is also dependent upon the type of peptide used, since enantiomers of polyarginine behave differently (Fretz et al. 2007). The biological cargo itself, including fluorescent conjugates, may also influence uptake and distribution by altering peptide interactions with the cell membrane or by changing intracellular peptide localization (Maiolo et al. 2005; Tunnemann et al. 2006). Cellular uptake may also be different for individual CPPs, resulting in peptide localization to different cellular compartments (Console et al. 2003). Some caution is warranted when assessing subcellular localization of cell-penetrating proteins or peptides because the majority of transduced molecules may be present in cytoplasmic vesicles, with only small quantities, possibly below the level of detection, localized to the site of biological activity (Tunnemann et al. 2006; Gump and Dowdy 2007).

Although the precise mechanisms of cellular entry for individual CPPs have not been well defined, efficacy of cell-penetrating peptides and proteins has been clearly demonstrated in a wide range of in vitro studies where these peptides and proteins have been used to dissect functions of a number of signaling molecules (for example, Hall et al. 2001; Myou et al. 2002; and Sano et al. 2005). These molecules have also shown promise in ameliorating pathogenesis in a variety of animal models of human disease, including allergic airways disease (Myou et al. 2003a, 2003b; Snyder and Dowdy 2005; Wadia and Dowdy 2005; Choi et al. 2006; McCusker et al. 2007).

### Cell-penetrating proteins as inhibitors of signaling pathways that contribute to eosinophil function in vitro and asthma pathogenesis in vivo

Central to the development and maintenance of asthma are T lymphocytes, which produce a number of mediators, including cytokines and chemokines, that increase recruitment of other inflammatory cells, such as eosinophils, to the lungs. While the precise role (or roles) of the eosinophil in asthma pathogenesis remains obscure (Jacobsen et al. 2007), abundant data demonstrate that eosinophils produce

a variety of mediators, such as cytokines, chemokines, leukotrienes, and bronchoconstrictors, which regulate airway inflammation, mucus production, and AHR (Wills-Karp 1999; Jacobsen et al. 2007). Recently, protein transduction technology has been used to define signaling pathways that regulate chemotaxis and survival of primary human eosinophils in vitro (Hall et al. 2001; Myou et al. 2002; Sano et al. 2005), processes that are important for the recruitment and maintenance of eosinophils in vivo.

Eosinophil chemotaxis, a process in which eosinophils roll, adhere, and migrate through the endothelium, relies upon integrin-mediated adhesion of eosinophils to ligands found on endothelial cells, including ICAM-1 and VCAM-1 (Nakajima et al. 1994; Broide and Sriramarao 2001; Wardlaw 2001). This process, particularly  $\beta_2$ -integrin-mediated eosinophil adhesion to ICAM-1, is regulated by extracellular signal-regulated kinase (ERK). Leff and colleagues used protein transduction technology to introduce cell-penetrating dominant negative forms of the Ras GTPase or the class Ia phospholipid kinase PI3K (phosphoinositide 3-kinase) into differentiated human eosinophils to assess the contribution of each of these signaling molecules to ERK activation and adhesion of eosinophils in culture (Myou et al. 2002; Sano et al. 2005). Introduction of either dominant negative Ras or dominant negative PI3K into these cells inhibited IL-5-induced activation of ERK kinases as well as the ability of eosinophils to adhere to ICAM-1-dependent substrata. In a similar study, Hall and colleagues demonstrated that IL-5-induced activation of Ras and ERK kinases as well as cell survival were impaired in cultured human eosinophils treated with a similar cell-penetrating dominant negative Ras protein (Hall et al. 2001).

These data demonstrate that PI3K and Ras regulate chemotaxis and survival of eosinophils in vitro. Because these signaling molecules also regulate responses in cultured airway structural cells as well as a number of inflammatory cells that participate in asthma pathogenesis, the ability of these cell-penetrating proteins to inhibit airway inflammation and AHR in murine models of allergic airways disease was assessed (Myou et al. 2003a, 2003b). Dominant negative forms of PI3K or Ras fused to the TAT PTD were injected intraperitoneally into antigen-sensitized recipient mice. Although the precise tissue distribution for each of these proteins was not presented, in vivo uptake into whole blood leukocytes was demonstrated by flow cytometry. Previous data have demonstrated uptake into the lung following intraperitoneal delivery of cell-penetrating proteins (Schwarze et al. 1999; Jo et al. 2005). When delivered intraperitoneally to mice throughout the antigen-challenge phase of an acute allergen sensitization and challenge protocol, each dominant negative protein inhibited antigen-induced airway inflammatory responses in a dose-dependent manner: bronchoalveolar lavage (BAL) fluid cell numbers, including eosinophils, were decreased and BAL fluid levels of IL-4 and IL-5 were inhibited. Antigen-induced increases in peribronchial inflammatory cells, mucus-containing epithelial cells, and AHR were also decreased in mice treated with each inhibitory protein. Intranasal administration of dominant negative Ras also effectively inhibited airway inflammation, assessed by quantifying inflammatory cells in the BAL fluid. Together, these data demonstrate that cell-

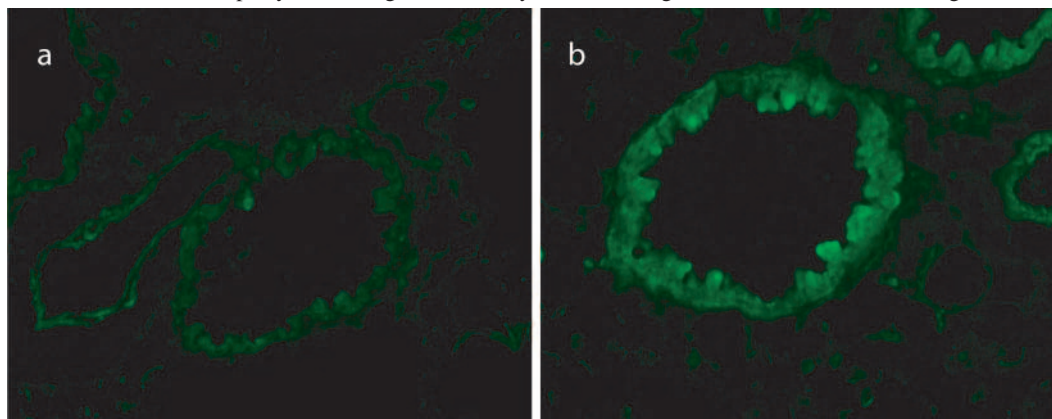
penetrating inhibitory proteins administered intraperitoneally as well as intranasally can effectively reduce airway inflammation, mucus production, and AHR in murine models of acute allergic airways disease.

### **Cell-penetrating peptides as inhibitors of inflammation and AHR in murine models of allergic airways disease**

We (McCusker et al. 2007) and others (Choi et al. 2006) have tested cell-penetrating peptides, as opposed to full-length proteins that target signaling pathways critical for T-cell activation or function, for their ability to inhibit allergic airways disease in mice. Lee and colleagues demonstrated that an arginine-rich sequence from the human Hph-1 transcription factor functioned as a PTD. Delivery of this PTD fused to peptide sequences from the carboxyl terminus of the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4) protein, which is a negative regulator of T-cell function, to human T cells in culture was shown to inhibit production of IL-2 and prevent T-cell activation (Choi et al. 2006). Moreover, intranasal delivery of this CTLA-4 fusion peptide inhibited the development of airway inflammatory responses, mucus production, and AHR in response to allergen challenge in mice. These data demonstrate that delivery of the CTLA-4 fusion peptide effectively inhibits inflammatory responses and AHR in a murine model of allergic airways disease. Interestingly, this CPP, which contains only 36 amino acids of the full-length CTLA-4 protein, does not appear to act in a dominant negative fashion, but instead retains inhibitory activity of the full-length CTLA-4 protein.

In our studies, we used protein transduction technology to target the signal transducer and activator of transcription factor-6, STAT-6 (McCusker et al. 2007). STAT-6 is activated by both IL-4 and IL-13, which share the IL-4 receptor alpha subunit in their cognate receptors (Hershey 2003). STAT-6 is required for IL-4 production from antigen-stimulated murine splenocytes (Kaplan et al. 1996). Moreover, activation of STAT-6 is critical for the differentiation of naïve T cells into Th2 effector cells, and STAT-6 regulates IL-4- and IL-13-induced production of Th2 chemokines, including eotaxin, from airway epithelial cells, fibroblasts, and smooth muscle cells (Ghaffar et al. 1999; Matsukura et al. 1999; Mathew et al. 2001; Matsukura et al. 2001; Chibana et al. 2003). Not surprisingly, STAT-6 is required for the development of airway inflammation, mucus production, and AHR in acute models of allergic airways disease (Kuperman et al. 1998; Mathew et al. 2001; Hoshino et al. 2004). We generated a cell-penetrating STAT-6 inhibitory peptide, STAT-6-IP (McCusker et al. 2007), consisting of the PTD4 protein transduction domain fused to a phosphotyrosine-containing sequence, that is predicted to bind with high affinity to the SH2 domain of STAT-6 and thus act as a dominant negative inhibitor of the wild-type STAT-6 protein. Our data demonstrate that the STAT-6-IP inhibits STAT-6-dependent production of IL-4 and IL-13 from antigen-stimulated primary murine splenocytes, as well as IL-4-induced eotaxin-3 production from cultured BEAS-2B epithelial cells. After intranasal delivery to nonallergic animals, the STAT-6-IP can be localized to airway epithelial cells (Fig. 1, and McCusker et

**Fig. 1.** To detect *in vivo* uptake of the STAT-6-IP, mice were briefly anaesthetized with halothane and then exposed intranasally to (a) saline or (b) 150  $\mu$ g fluorescein-conjugated STAT-6-IP in a volume of 40  $\mu$ L. Four hours later, the mice were killed and the lungs lavaged with 2 mL PBS, then harvested and fixed in 2% paraformaldehyde. Immunofluorescence of lung sections was detected by using an Olympus BX 51 fluorescence microscope system. Images were analyzed with Image-Pro Plus 4.5 software. Magnification  $\times$  40.



al. 2007). Significantly, intranasal delivery of the STAT-6-IP before antigen challenge decreases airway eosinophilic inflammation, eotaxin expression, mucus production, and AHR in murine models of experimental rhinitis and asthma induced by either ovalbumin or ragweed (McCusker et al. 2007), (data not shown). Although we can detect the STAT-6-IP in airway epithelial cells (Fig. 1), it is not clear whether inhibition of STAT-6 activity in these cells is sufficient to ameliorate inflammation and AHR in mice treated with the STAT-6-IP. Thus, further studies need to be undertaken to define mechanism(s) by which the STAT-6-IP inhibits allergic airways disease in these models.

In addition to STAT-6, other STAT factors may also participate in allergic inflammatory processes. For example, IL-4-dependent Th2 cell differentiation is regulated by STAT-5. Nakayama and colleagues demonstrated that a chimeric peptide containing the penetratin CPP conjugated to a phosphotyrosine-based STAT-5 $\alpha$ -binding peptide, which is predicted to prevent activation and (or) dimerization of STAT-5, significantly inhibited *in vitro* Th2 cell development from murine splenocytes (Yamashita et al. 2000). These data suggest that *in vivo* delivery of the STAT-5-blocking peptide may also ameliorate allergic airways disease.

Another important transcriptional regulator of T-cell responses is NF $\kappa$ B (Barnes and Karin 1997; Mercie et al. 1998). Data obtained by using transgenic mice in which NF $\kappa$ B activity is disrupted in airway epithelial cells demonstrate that NF $\kappa$ B regulates airway inflammation and remodeling in murine models of allergic airways disease (Poynter et al. 2004; Broide et al. 2005). Although the precise proteins whose expression is modified by NF $\kappa$ B in these models are poorly defined, NF $\kappa$ B is required for expression of GATA-3 (Das et al. 2001), a Th2 cell-specific transcription factor that induces expression of IL-4, IL-5, and IL-13 (Zheng and Flavell 1997). NF $\kappa$ B consists of homo- or heterodimers of the p50, p65, p52, relB, or c-rel subunits localized to the cytosol and complexed with I $\kappa$ B inhibitory proteins. When phosphorylated, I $\kappa$ B is targeted for ubiquitination and proteasome-dependent degradation. Subsequently, NF $\kappa$ B translocates to the nucleus and regulates gene expression. Translocation of NF $\kappa$ B to the nucleus is mediated through its nuclear localization sequence (NLS)-

dependent interaction with karyopherin proteins (Hurt 1996; Mercie et al. 1998). Data from Fujihara and colleagues demonstrated that a chimeric peptide containing a CPP from the fibroblast growth factor and 2 NLS sequences predicted to bind to karyopherin- $\alpha$  disrupted the NF $\kappa$ B:karyopherin interaction. This peptide inhibitor of NF $\kappa$ B translocation binds to and displaces NF $\kappa$ B, thereby preventing nuclear localization of NF $\kappa$ B (Fujihara et al. 2000) and NF $\kappa$ B-dependent cell proliferation. When utilized in an LPS-induced mouse model of inflammatory bowel disease, the peptide inhibitor of NF $\kappa$ B reduced morbidity and increased levels of circulating IL-10, an immunoregulatory cytokine (Grutz 2005). Although efficacious in immunomodulation, this peptide does not specifically target NF $\kappa$ B and can potentially prevent nuclear localization of any protein that binds to karyopherin- $\alpha$ .

A more specific peptide inhibitor of NF $\kappa$ B was recently developed by conjugating a PTD to the C terminus of I $\kappa$ B (I $\kappa$ B $\alpha$ - $\Delta$ N) to produce a chimeric peptide that effectively displaces endogenous I $\kappa$ B molecules and is refractory to phosphorylation (Mora et al. 2005). In the absence of phosphorylation, the NF $\kappa$ B-I $\kappa$ B complex remains in the cytoplasm, thereby preventing NF $\kappa$ B activation and nuclear translocation. Treatment of T cells with the I $\kappa$ B inhibitory peptide prevents antigen-induced T-cell proliferation *in vitro* (Mora et al. 2005). This I $\kappa$ B inhibitory peptide also effectively prevents NF $\kappa$ B activation in 2 animal models of inflammation: skin wounding in mice and endotoxin treatment in sheep. Interestingly, the mainstay for the treatment of allergic airway diseases, glucocorticoids, inhibits NF $\kappa$ B activation (Scheinman et al. 1995). Thus, while not yet tested in animal models, cell-penetrating peptides targeting the NF $\kappa$ B pathway may also provide a new therapeutic option for the treatment of allergic airways disease.

## Concluding remarks

Altogether, these data demonstrate that CPPs, when conjugated to biologically active molecules, can be used to dissect signaling pathways in eosinophils and other difficult-to-transfect cells implicated in asthma pathogenesis. These data also demonstrate that CPPs can be used to assess the role played by signaling molecules and other proteins, for

which pharmacological inhibitors may not be available, in allergic airways disease. The ability of cell-penetrating proteins and peptides to inhibit inflammation in murine models of allergic airways disease is promising. Nevertheless, several important questions remain. While we and others have demonstrated uptake of cell-penetrating proteins and peptides, delivered either intranasally or intraperitoneally, by BAL fluid cells, airway epithelial cells, and lung CD3<sup>+</sup> T cells, in-depth studies monitoring distribution and stability in the upper and lower airways have not been described. As well, the ability of these inhibitory proteins or peptides to diminish airway remodeling in chronic models of allergic airways disease needs to be assessed, and additional studies in large animal models need to be undertaken. Nevertheless, data from murine models are promising and suggest that cell-penetrating peptides or proteins may have a future in the treatment or prevention of allergic airways disease in humans.

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