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Cell-Surface Peptidases*

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The cell surface has various functions: communicating with other cells, integrating into the tissue, and interacting with the extracellular matrix. Proteases play a key role in these processes. This review focuses on cell-surface peptidases (ectopeptidases, oligopeptidases) that are involved in the inactivation or activation of extracellular regulatory peptides, hormones, paracrine peptides, cytokines, and neuropeptides. The nomenclature of cell-surface peptidases is explained in relation to other proteases, and information is provided on membrane anchoring, catalytic sites, regulation, and, in particular, on their physiological and pharmacological importance. Furthermore, nonenzymatic (binding) functions and participation in intracellular signal transduction of cell surfaces peptidases are described. An overview on the different cell-surface peptidases is given, and their divergent functions are explained in detail. An example of actual pharmacological importance, dipeptidyl-peptidase IV (CD26), is discussed.

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I. Introduction

The cell surface is the site for communication of cells with each other or with the whole body, integration of cells into their tissue, and modeling of the tissue matrix. Some of these diverse functions are regulated by proteases that cleave and thereby may activate, inactivate, or release other proteins or peptides. These regulatory proteases are either located on the cell surface

*Dedicated to Professor Dr. Eberhard Heymann on the occasion of his 65th birthday.

or are secreted by the cells. Both types of proteases may fulfill similar functions, but cell-surface proteases appear to have in general more regulatory and specialized functions than secreted ones. In particular, they are involved in the regulation of the activity of peptides that communicate between cells or tissues (**oligopeptidases**, **cell-surface peptidases** in the original sense), in the degradation of extracellular components (**matrix proteases**), or in the shedding of cell-surface proteins (**secretases**, **sheddases**).

Since this comprises a broad spectrum of different types of proteases this review focuses on cell-surface (oligo)peptidases ([Table I](#)), a special group of cell-surface proteases that is involved in the regulation of the biological activity of small bioactive peptides that serve in cell communication, namely peptide hormones, neuropeptides, growth factors, or cytokines. These important physiological roles have focused attention on cell-surface peptidases in recent pharmacological research. Inhibitors are widely used as drugs or are under development. This review will provide an overview of the general properties and physiological and pharmacological importance of mammalian cell-surface (oligo)peptidases. A closer view will be given to a selected peptidase of actual pharmacological interest, the dipeptidyl-peptidase IV.

Other types of cell-surface proteases act mainly on larger protein/proteoglycan substrates. Membrane-type matrix metalloproteinases (MT-MMPs) belong to the group of matrix metalloproteinases (MMPs) that is primarily involved in the degradation of extracellular matrix components. MMPs are mostly secreted, and cell-surface MT-MMPs either act like the secreted MMPs or activate them. Secretases or sheddases are proteases that release soluble forms from transmembrane proteins or peptides, e.g., cell-surface receptors, membrane anchored cytokines or chemokines, other membrane proteases, or the amyloid precursor protein. They belong to the group of ADAMs (a disintegrin and metalloproteinase). ADAMs may cleave and release other cell-surface peptidases to yield soluble forms. Since MT-MMPs and ADAMs both act on larger proteins and belong either to a broader group of secreted proteases (MMPs) or represent a distinct group from the (oligo)peptidases, they will be excluded from this review (the reader is referred to specialized reviews for more information: [Itoh and Nagase, 2002](#); [Moss and Lambert, 2002](#); [Visse and Nagase, 2003](#); [Zucker and Chen, 2003](#)).

Interestingly, many cell-surface (oligo)peptidases—though specialized on short peptides—belong to families of proteins that have a broader spectrum of activities, not only as peptidases for regulatory peptides, but also as proteases for larger proteins, e.g., extracellular matrix proteins or proteoglycans, or even as binding proteins for these molecules without any proteolytic activity. Therefore it is not surprising that some cell-surface peptidases may have multiple functions, as a regulatory peptidase, extracellular protease, or a binding protein. These diverse functions will be discussed in this review in terms of the cell-surface peptidase dipeptidyl-peptidase IV (CD26) and

TABLE I
Overview of Cell-Surface Peptidases

| Peptidase | Abbreviation | EC | Catalytic type | Alternative names (examples) |
|-----------------------------------|----------------------|-----------|----------------|--|
| Aminopeptidase N | APN (CD13) | 3.4.11.2 | Metallo | Alanine aminopeptidase, aminopeptidase M, microsomal aminopeptidase, Cys-Gly dipeptidase |
| Aminopeptidase A | APA | 3.4.11.7 | Metallo | Angiotensinase A, gp160, glutamyl aminopeptidase, membrane aminopeptidase II, mouse BP-1/6C3 antigen |
| Aminopeptidase P | APP | 3.4.11.9 | Metallo | Aminopeptidase P2, membrane aminopeptidase P, proline aminopeptidase, X-Pro aminopeptidase |
| X-Trp aminopeptidase | | 3.4.11.16 | Metallo | Aminopeptidase W |
| Pyroglutamyl-peptidase II | | 3.4.19.6 | Metallo | TRH-degrading ectoenzyme, thyroliberinase |
| Dipeptidyl-peptidase IV | DPP IV (CD26) | 3.4.14.5 | Serine | Gly-Pro naphthylamidase, Xaa-Pro-dipeptidyl-aminopeptidase, adenosine deaminase binding protein, glycoprotein GP110, thymocyte-activating molecule |
| Angiotensin-converting enzyme | ACE (CD143) | 3.4.15.1 | Metallo | Peptidyl-dipeptidase A, dipeptidyl carboxypeptidase I, kininase II |
| Angiotensin-converting enzyme-2 | ACE2, ACEH | | Metallo | |
| Carboxypeptidase M | CPM | 3.4.17.12 | Metallo | Max. I |
| Carboxypeptidase P | CPP | 3.4.17.16 | Metallo | Membrane Pro-X carboxypeptidase |
| γ -Glutamyl transpeptidase | γ -GT (CD224) | 2.3.2.2 | | γ -Glutamyltransferase |
| Membrane dipeptidase | MDB | 3.4.13.19 | Metallo | Leukotriene D4 hydrolase, renal dipeptidase |
| Nepilysin | NEP (CD10) | 3.4.24.11 | Metallo | Neutral endopeptidase, endopeptidase 24.11, enkephalinase, atriopепtidase, CALLA ^a |
| Endothelin-converting enzyme | ECE-1 | 3.4.24.71 | Metallo | |

^aCALLA, common acute lymphoblastic leukemia antigen.

the members of its family, since this cell-surface peptidase is of actual pharmacological importance.

Today, the properties, structures, distribution, and physiological and pharmacological importance of most cell-surface peptidases are well described, but less is known about their role in cell biology. For example, when a cell-surface peptidase is a multimeric enzyme, it is rarely explored whether it can form heteromers with another member of its gene family resulting in further functions. Also relatively little is known about the dynamics of the peptidases on the cell surface, their association with other proteases, receptors, transporters, or binding proteins, and their fate during endocytotic processes.

In this review I will provide a general overview of cell-surface (oligo)-peptidases (Table I), in particular their nomenclature, structure, cell anchoring, and physiological and pharmacological importance. These properties will then be discussed in terms of a selected surface peptidase with actual pharmacological interest.

II. Classification and General Properties of Cell-Surface Peptidases

A. Nomenclature and Classification

Enzymes hydrolyzing peptide bonds are commonly termed **proteases**, **proteinases**, **peptidases**, or **proteolytic enzymes**. Historically, these terms have had slightly different meanings, namely peptidases act on small peptides, proteases degrade larger proteins, and proteinases (endopeptidases) cleave peptide chains internally (Barrett and McDonald, 1980; McDonald and Barrett, 1986), but these terms are now often used synonymously. The Enzyme Nomenclature EC list (1992) (<http://www.chem.qmw.ac.uk/iubmb/enzyme/index.html>) recommends the term *peptidase* as the general form. Here, the term **cell-surface peptidase** is used in the former sense for a proteolytic enzyme acting on small peptides (now oligopeptidase) that is located at the cell surface with an extracellular active site (for which the general term *ectoenzyme* is used).

Exopeptidases cleave only one or a few residues from the N- or C-terminus of a peptide, whereas *endopeptidases* (formerly identical to proteinases) act internally in a peptide chain. *Aminopeptidases* liberate either a single amino acid residue from the N-terminus (*aminopeptidases*, EC 3.4.11) or a dipeptide or tripeptide from the N-terminus (*dipeptidyl-peptidases* and *tripeptidyl-peptidases*, EC 3.4.14). Those cleaving a single residue from the C-terminus are termed *carboxypeptidases* (EC 3.4.16–18) and those liberating a dipeptide are termed *peptidyl-peptidases* (EC 3.4.15). Other exopeptidases

are specific for dipeptides (*dipeptidases*, EC 3.4.13), or remove terminal residues that are substituted, cyclized, or linked to isopeptide bonds (peptide linkage at amino acid side chains); the later are termed *omega peptidases* (EC 3.4.19). The term *oligopeptidase* is also used for endopeptidases that act optimally on smaller peptides, not on proteins. Cell-surface peptidases acting on small peptides belong to all groups (endopeptidases, exopeptidases, and specialized peptidases); those acting on larger proteins are endopeptidases.

Proteases are further classified according to their catalytic center into serine, cysteine, aspartic, or metalloproteases (Barrett, 1994). Cell-surface peptidases are either serine or (more often) metalloproteases. Based on molecular structure and evolutionary relationship they can be divided into families and clans. This system is explained in detail by Barrett *et al.* (1998) and is used in the MEROPS database (<http://merops.sanger.ac.uk/>). This database gives excellent, precise, and detailed information about proteases of many species. In this scheme, a *family* of peptidases is a group in which each member shows a statistically significant relationship in amino acid sequence to at least another member of the family. The term *clan* is used to describe a group of families the members of which have evolved from a single ancestral protein, but have diverged so far that their relationship cannot be proved by direct comparison of their primary structures.

Apart from the excellent MEROPS database (<http://merops.sanger.ac.uk/>), information on the protein structures of proteases can be obtained from the SwissProt protein sequence database (<http://www.expasy.ch/>) or especially for enzymes from <http://www.expasy.org/enzyme/>, or the PIR database (<http://pir.georgetown.edu/>). Genetic information can be obtained from <http://www.ncbi.nlm.nih.gov/> or <http://bioinformatics.weizmann.ac.il/cards/> or from <http://www.dsi.univ-paris5.fr/genatlas/>. The catalytic properties of proteases are compiled in the Brenda enzyme information system (<http://brenda.bc.uni-koeln.de/>); further information and especially animations are given at <http://delphi.phys.univ-tours.fr/Prolysis/>. An excellent print compilation of proteases is given in the “Handbook of Proteolytic Enzymes” (Barrett *et al.*, 1998). For older references, the compilations of Barrett and McDonald (1980), McDonald and Barrett (1986), Kenny (1977), Kenny and Hooper (1991), and Kenny *et al.* (1987) are still valuable sources.

B. Membrane Anchoring of Cell-Surface Peptidases and Soluble Forms

In contrast to intracellular or extracellular secreted proteases, cell-surface peptidases have to be anchored into the plasma membranes. They are nearly exclusively integral membrane proteins, but there are a few examples that soluble, secreted proteases can also be bound to the cell surface via receptors.

For example, the secreted, soluble urokinase (urokinase-type plasminogen activator, EC 3.4.21.73), a protease involved in the activation of other proteases, clot-lysis, and cell invasion, is bound to the cell surface via a specific membrane receptor. As integral membrane proteins, cell-surface peptidases either possess a transmembrane region with the N- (type I) or C-terminus (type II) extracellular, or they are linked to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor (Fig. 1). As cell-surface proteins they are *glycoproteins* with a high degree (e.g., of about 30% of their molecular mass) of carbohydrates that are found at the extracellular site.

Some cell-surface peptidases also exist in soluble forms. These soluble forms are apparently generated by proteolytic shedding from the cell surface (Fig. 1) rather than being different splice forms. So far, the conditions for their shedding and the secretases involved have rarely been investigated. An exception is angiotensin-converting enzyme (ACE; EC 3.4.15.1) from which a soluble form is known to be liberated by members from the ADAM family (Hooper *et al.*, 1997; Parkin *et al.*, 2002). Soluble ACE (as some other soluble forms of cell-surface peptidases) is present under normal physiological conditions in blood plasma, amniotic fluid, seminal plasma, and other body fluids. The levels of soluble ACE are altered in certain diseases such as sarcoidosis, diabetes mellitus, Gaucher's disease, leprosy, and hyperthyroidism.

However, soluble equivalents to cell-surface peptidases also exist that are encoded by different genes. Aminopeptidase P (APP, X-Pro aminopeptidase, EC 3.4.11.9) has two forms: a membrane-bound form (AP-P2) encoded by a gene on the human X chromosome (Sprinkle *et al.*, 1998) and a cytosolic form (AP-P1) encoded by a gene on human chromosome 10 (Sprinkle *et al.*, 2000), both with nearly identical substrate specificities. The membrane-bound form

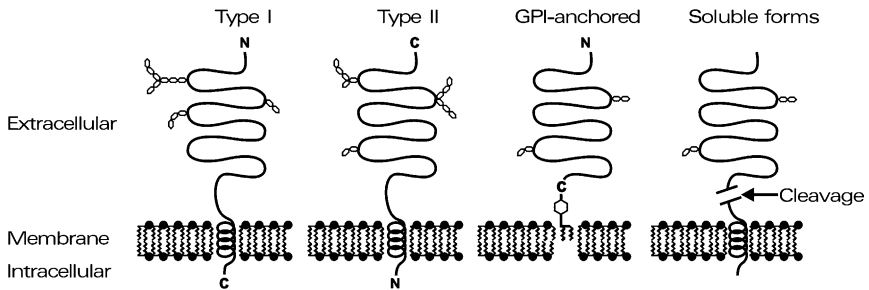


FIG. 1 Topology of cell-surface peptidases. Cell-surface peptidases are anchored in the plasma membrane either with a transmembrane region (schematic: lipid bilayer with helical lipophilic amino acid sequences; type I = N-terminus extracellular; type II = C-terminus extracellular), or they are linked to the membrane via a glycosyl-phosphatidylinositol (GPI) anchor. The extracellular region is highly glycosylated (schematic: hexoses). Secretases may liberate the extracellular parts of cell-surface peptidases to yield soluble variants.

is located as an ectoenzyme (via a glycosyl-phosphatidylinositol anchor) on the plasma membrane of endothelial and epithelial cells whereas the soluble form is a cytosolic enzyme found in leukocytes, platelets, and brain (Harbeck and Mentlein, 1991; Hooper *et al.*, 1990; Simmons and Orawski, 1992). The cytosolic form displays 43% amino acid identity and 63% similarity to the membrane-bound form (Cottrell *et al.*, 2000) and both are classified as members of the peptidase clan MG on the basis of sequence and structural similarities (Hyde *et al.*, 1996) with other exopeptidases, which include the soluble peptidases such as methionyl aminopeptidases type I and II (EC 3.4.11.18) and the X-Pro dipeptidases (prolidases, EC 3.4.13.9).

C. Catalytic Sites and Kinetics of Cell-Surface Peptidases

Since cell-surface peptidases act on extracellular regulatory peptides, the active catalytic site is located in their large extracellular domain. Therefore, cell surface peptidases are also termed *ectopeptidases*. According to catalytic type, cell-surface peptidases possess either a catalytic triad of His, Asp, and Ser (serine peptidases) or a metallo-binding motif for a divalent cation, normally zinc (metalloproteases). The known metal ligands in metalloproteases are His, Glu, Asp, or Lys. In addition to the metal ligands, at least one other residue is required for catalysis, mostly Glu, but also Arg and Lys. Normally, the catalytic site is found only in one catalytic center, but, e.g., angiotensin-converting enzyme (EC 3.4.15.1) can have two catalytic HEXXH metalloprotease motifs (Barrett *et al.*, 1998; Hooper, 1994). The three-dimensional structure of some cell-surface peptidases (metallo- and serine peptidases) has been clarified in some examples of pharmacological importance, and the reader is referred to original and more specialized review articles (Engel *et al.*, 2003; Lowther and Matthews, 2002; Natesh *et al.*, 2003; Rasmussen *et al.*, 2003).

The specificity of the cleavage for peptide bonds by cell-surface peptidases can be described with the terminology of Berger and Schechter (1970). The residues to the peptide substrate are numbered according to (N-terminus) . . . P₃-P₂-P₁ + P_{1'}-P_{2'}-P_{3'} . . . (C-terminus; + marks the cleavage site). Some cell-surface peptidases show exceptionally high cleavage selectivity, e.g., APP liberates only P₁ residues from the N-terminus of peptides with a proline in the P_{1'} position; others show a much broader selectivity.

The Michaelis-Menten kinetics of enzymes is normally characterized by K_m and V_{max} (respectively $k_{cat} = V_{max}/\text{mol enzyme}$) values. However, K_m values for oligopeptidases are in the micromolar range whereas the physiological concentrations of regulatory peptides (hormones, neuropeptides, cytokines) are in the nanomolar or picomolar range (as the K_d values of their receptors). At these low concentrations of substrates S (in comparison

to K_m values) the reaction rates of the enzymes E are given by $v = [E][S] k_{cat}/K_m$, i.e., the linear curve of dependence of v from S (Fersht, 1985). Therefore, the rate (specificity) constant k_{cat}/K_m is the best basis for comparing the potency of a peptidase with different peptides at physiological concentrations. High rate constants correspond to high cleavage rates at low (physiological) concentrations.

D. Cell-Surface Proteases and CD Nomenclature

Certain cell-surface molecules, including some cell-surface peptidases, have been identified on immune cells by the use of monoclonal antibodies (initially without knowing their broader tissue distribution). Based on this differentiation by monoclonal antibodies, cluster of differentiation (CD) antigens have been defined that have later been shown to be identical to some well-studied cell-surface peptidases (Table II). For example, CD10 was originally described as CALLA (common acute lymphoblastic leukemia antigen), because of its association with ALL (acute lymphoblastic leukemia) and its usefulness in diagnosis, and has been identified later as the already known neutral endopeptidase 24.11 (neprilysin, enkephalinase, EC 3.4.24.11). Further information on CD antigens, their nomenclature, distribution, and function can be found at <http://www.ncbi.nlm.nih.gov/PROW/>.

TABLE II
Cell-Surface Peptidases in the CD Nomenclature

| CD | Alternative names | Peptidase | EC |
|------------------|--|---|-----------|
| CD10 | CALLA ^a | Neutral endopeptidase (NEP), neprilysin, enkephalinase | 3.4.24.11 |
| CD13 | | Aminopeptidase N (APN) | 3.4.11.2 |
| CD26 | Adenosine deaminase-binding protein, THAM ^b | Dipeptidyl-peptidase IV (DPP IV) | 3.4.14.5 |
| CD143 | | Angiotensin-converting enzyme (ACE), peptidyl dipeptidase A | 3.4.15.1 |
| CD224 | | γ -Glutamyl transpeptidase (γ -GT) | 2.3.2.2 |
| Max.1 | | Carboxypeptidase M (CPM) | 3.4.17.12 |
| BP-1/6C3 antigen | | Aminopeptidase A (APA) | 3.4.11.7 |

^aCALLA, common acute lymphoblastic leukemia antigen.

^bTHAM, thymocyte activation molecule.

III. Physiological Functions and Pharmacological Importance of Cell-Surface Peptidases

Initially, cell-surface proteases were isolated from kidney or intestinal brush border membranes, both rich sources of membrane-bound proteases (Kenny, 1977; Kenny *et al.*, 1987). These locations pointed to a physiological role in the final degradation of peptides or peptide fragments produced by other proteases. In fact, cell-surface proteases act mostly on substrates with less than 80 amino acid residues and some exhibit broader substrate specificities (Table III). Therefore, it can be anticipated that cell-surface peptidases on the intestinal, luminal surface are involved in the digestion of nutritional proteins. This digestive role has been shown for cell-surface proteases of broad specificity such as aminopeptidase N as well as for proline-specific peptidases such as dipeptidyl-peptidase IV or carboxypeptidase P (Brandsch *et al.*, 1995; Erickson *et al.*, 1989; Heymann and Mentlein, 1986; Yoshioka *et al.*, 1988). Since peptide bonds involving proline residues are resistant to the attack of nonspecific proteases such as trypsin, chymotrypsin, aminopeptidases, or carboxypeptidases, the proline-specific exopeptidases appear to be necessary for the final digestion of peptides. However, so far knockout or deficient animals show no clinical abnormalities in protein digestion (see below, Table IV); however, a case of (probable) human aminopeptidase P deficiency showed peptiduria of Xaa-Pro-peptides (Blau *et al.*, 1988).

Later, cell-surface peptidases with very narrow specificity such as pyroglutamyl-peptidase II or endothelin-converting enzyme were detected. Furthermore, it was discovered that all cell-surface peptidases acted on regulatory peptides, and even those with an intestinal location turned out to be located on more discrete sites where they have contact with endogenous hormones or neuropeptides. As a consequence, the degradation of bioactive peptides or other cellular (binding) functions is regarded as the main function of cell-surface peptidases.

A. Inactivation and Activation of Regulatory Peptides and their Pharmacological Importance

Proteolytic processing is involved in the inactivation, partial inactivation, or activation of regulatory peptides such as hormones, neuropeptides, or cytokines/chemokines. Activation of intracellular stored peptides is normally accomplished by signal peptidases and prohormone convertases within the secretory vesicles. Extracellular proteolytic activation occurs for some secreted hormones (e.g., angiotensinogen/angiotensin) or for the conversion of membrane-bound forms to active soluble forms (e.g., tumor necrosis

TABLE III
Physiological Substrates of Cell-Surface Peptidases

| Peptidase | Substrate specificity | Endogenous substrates | Characteristic inhibitors |
|--|-------------------------------|---|---|
| APN (CD13) EC 3.4.11.2 | Xaa-↓-Xaa- | Enkephalins, neurokinin A, somatostatin | Bestatin, actinonin, probestin, amastatin |
| APA EC 3.4.11.7 | Glu/Asp-↓-Xaa... | Angiotensin II | Amastatin |
| APP EC 3.4.11.9 | Xaa-↓-Pro- | Bradykinin, neuropeptide Y | Apstatin |
| Pyroglutamyl- peptidase II EC 3.4.19.6 | Glp-↓-His-Pro-NH ₂ | TRH | 1,10-Phenanthroline (nonselective) |
| DPP IV (CD26) EC 3.4.14.5 | Xaa-Pro/Ala-↓-Yaa | See Table IV | Diprotin A, Xaa-thiazolides, Xaa-pyrrolidides, Xaa-boroPro |
| CPM EC 3.4.17.12 | ...Xaa-↓-Arg/Lys | Bradykinin, anaphylatoxins, EGF | MGTA, GEMSA ^a |
| CPP EC 3.4.17.16 | ...Pro-↓-Xaa | Angiotensin II, enterostatin | Phenanthroline (nonselective) |
| ACE (CD143) EC 3.4.15.1 | ...Xaa-↓-Yaa-Zaa | Angiotensin II, bradykinin, enkephalins | Captopril, enalapril, lisinopril |
| ACE-2 | ...Xaa-↓-Yaa-Zaa | Angiotensin II | Metal chelators |
| MDB(-1) EC 3.4.13.19 | Dipeptides | Peptidyl leukotriene D ₄ β-lactam antibiotics | Cilastatin |
| NEP (CD10) EC 3.4.24.11 | ...Xaa-↓-Yaa | Enkephalins, bradykinin, substance P, neurotensin, atrial natriuretic factor, gonadoliberin, calcitonin | Phosphoramidon, thiorphan, omapatrilat (mixed inhibitor) |
| ECE-1 EC 3.4.24.71 | | Big endothelin | Phosphoramidon, PD 069185 |

^aMGTA, 2-mercaptomethyl-3-guanidinoethylthiopropionic acid; GEMSA, guanidinoethylmercaptosuccinic acid.

TABLE IV

Phenotype of Knockout or Deficient Animals for Cell-Surface Peptidases

| Peptidase | Phenotype | References |
|---------------|--|--|
| APN (CD13) | Dogs with deficiency in small intestine were clinically healthy | Pemberton <i>et al.</i> , 1997 |
| APA | Mice with deficiency developed normally, normal numbers of T and B cells, normal antibody responses and IgG | Lin <i>et al.</i> , 1998 |
| APP | Candidate gene for premature ovarian failure | Prueitt <i>et al.</i> , 2000 |
| DPP IV (CD26) | Knockout mice are refractory to obesity and hyperinsulinemia after high-fat diet, have higher glucose tolerance and GLP-1 levels | Conarello <i>et al.</i> , 2003 |
| | Higher glucose tolerance in knockout mice or rats with mutated enzyme | Marguet <i>et al.</i> , 2000; Mitani <i>et al.</i> , 2002; Nagakura <i>et al.</i> , 2001 |
| | Reduced tumor metastasis in rats with mutated enzyme | Shingu <i>et al.</i> , 2003 |
| ACE (CD143) | Knockout mice: reduced blood pressure, renal pathology, and reduced male fertility | Esther <i>et al.</i> , 1996 |
| NEP (CD10) | Knockout mice: sensitivity to endotoxin shock, opioid-related increase in thermociceptive threshold, modified ventilatory response to hypoxia, higher response to experimentally induced colitis. Overexpression: reduced deposition of β -amyloid | Lu <i>et al.</i> , 1995; Maar <i>et al.</i> , 2003; Saria <i>et al.</i> , 1997; Sturiale <i>et al.</i> , 1999 |
| ECE-1 | Null mutation in mice results in embryonic craniofacial and cardiac abnormalities | Yanagisawa <i>et al.</i> , 1998 |
| | Mutations in humans are associated with Hirschsprung disease | Hofstra <i>et al.</i> , 1999 |
| MDB-1 | Knockout mice: no phenotype, redundant enzymes exist | Habib <i>et al.</i> , 1998 |

factor- α , fractalkine). Whereas in the later case the soluble forms are generated mostly by members of the ADAM family from the integral membrane proforms, secreted prohormones are cleaved by cell-surface proteases (or their shedded soluble forms) or proteases of broader specificity.

For *activation* of regulatory peptides by cell-surface proteases the prominent examples are ACE, which activates angiotensin I to the potent vasopressor octapeptide angiotensin II by release of a C-terminal dipeptide, and endothelin-converting enzyme (ECE), which cleaves big endothelin to endothelins (Table III). Whereas ACE displays a relatively broad specificity (in addition to angiotensin I bradykinin, neurotensin, substance P, and gonadoliberin are also cleaved) and is implicated in blood pressure regulation

as well as a range of further biological processes such as immunity, reproduction, and neuropeptide regulation, ECE is a more specific enzyme that activates (inactive) big endothelin to the active vasoconstrictor endothelin(s). Consequently, inhibitors of ACE or ECE reduce the formation of the active, hypertensive hormones angiotensin II or endothelins and are therefore used as antihypertensive drugs (for details on ACE or ECEs see below, [Barrett et al., 1998](#); or the MERPOS database at <http://merops.sanger.ac.uk/>).

Partial inactivation/activation is a process by which regulatory peptides are processed to truncated forms that retain only physiological activity to one receptor subtype or create activity to a new one. Since this differential inactivation often depends on N- or C-terminal residues, exopeptidases are mostly involved in this process. Detailed examples are discussed below for peptide truncation by dipeptidyl-peptidase IV (DPP IV), but similar partial inactivation of regulatory peptides is also observed for aminopeptidase P, carboxypeptidase M (bradykinin), and aminopeptidase A (forming angiotensin III).

Sometimes truncated peptides still bind to their receptors but do not transduce signals into their target cells. Consequently, cell-surface peptidases can generate antagonists. Their affinity is normally less or equal to the native peptide, and, therefore, it is doubtful that production of antagonists is of high physiological relevance *in vivo*. However, these proteolytically generated antagonists can down-regulate the surface expression of their receptors. This is of crucial importance for receptors with high internalization rates, e.g., chemokine receptors. Here, cell surface peptidases (DPP IV, see below) create antagonists with reduced affinity, but still important receptor regulation properties ([Ludwig et al., 2002](#)).

Inactivation of regulatory peptides is regarded as the main physiological function of cell-surface peptidases. This role concerns peptide hormones circulating in the blood stream, paracrine/autocrine hormones in tissues, and neuropeptides in the brain (or peripheral sites). Inactivation is a necessary event for regulated signal transmission, otherwise only permanent stimuli would result. The half-lives of signal molecules for extracellular communication differ considerably: from milliseconds to seconds for “classical” neurotransmitters (acetylcholine, amino acids, catecholamines), seconds to minutes for neuropeptides and peptide hormones, and minutes to hours or even days for growth factors and cytokines. Proteolytic degradation or other chemical modifications (for peptides oxidation or conjugation reactions occur in rare examples, but with questionable physiological relevance) are not the only inactivation mechanism; elimination by diffusion or filtration is also important (high-affinity cellular uptake of peptides is not observed). For example, a 30-residue peptide hormone such as glucagon-like peptide-1 (GLP-1, see below) circulates in the blood for 30–60 min after postprandial release from the intestine; during this time it is removed by filtration into the

urine by the kidney. However, inactivation by proteolytic truncation (in this case by DPP IV, see below) considerably reduces the active form during its physiological activity as an insulin releaser. Consequently, cell-surface peptidase inhibitors are potential drugs that prolong the activity of endogenous regulatory peptides (or, in case of activation, hinder their generation).

Table III lists some important physiological substrates for cell-surface peptidases. Common for all peptidases is their restriction to peptide substrates up to about 80 residues (therefore the term oligopeptidases is adequate). Some of them exhibit a rather broad specificity, e.g., aminopeptidase N (APN) and neprilysin (NEP), others are restricted to certain types of bonds that are cleaved, e.g., APP and DPP IV, and still others are substrate specific, namely pyroglutamyl-peptidase II and (not strictly) ECE. If a certain regulatory peptide is cleaved by different cell-surface peptidases, e.g., substance P, bradykinin, and enkephalins, it depends on the tissue/cellular environment, which for the cell-surface proteases are of crucial importance. Methods to determine the degradation of regulatory peptides often include cell culture systems or purified systems, and the degradation is monitored now by high-performance liquid chromatography (HPLC) with reversed-phase separation columns using gradients of acetonitrile in 0.1% trifluoroacetic acid, capillary electrophoresis, or mass spectroscopy (Mentlein and Lucius, 1997).

Interestingly, although a peptidase may cleave a variety of peptides, inhibitors can target mainly one system in a certain disease. Inhibitors of ACE (inhibition of angiotensin II formation), ECE (inhibition of endothelin formation), and NEP (prolongation of the actions of atrial natriuretic peptides) are used to reduce blood pressure, DPP IV inhibitors (prolongation of GLP-1 action, see below) are under development for the treatment of type 2 diabetes, and most of the targets (ACE, NEP, and DPP IV) also cleave various other regulatory peptides.

The following sections contain a general overview of different cell-surface peptidases and their roles in the inactivation or activation of regulatory peptides. This is restricted to important examples. In one example of actual pharmacological importance (DPP IV) a detailed description is given at the end that summarizes not only its diverse functions as a regulatory protease, but also provides information on further members of its protein family and illustrates additional, nonproteolytic functions of cell-surface peptidases.

1. Aminopeptidases

Aminopeptidase N can attack a variety of substrates (up to 40 residues), although hydrophobic and neutral residues are preferred. Enkephalins/dynorphins appear to be the most important substrates. It should be noted that truncation of some regulatory peptides (e.g., somatostatin) does not

inactivate them. The enzyme (homodimer of highly glycosylated, type II integral membrane 140–150 kDa subunits) is found in the brush border membranes of kidney, intestinal mucosal cells, and liver. Important localizations for the catabolism of regulatory peptides are endothelial cells (apical domain), smooth muscle cells, monocytes/macrophages (or related cells, e.g., microglial cells), and granulocytes (Barrett *et al.*, 1998; Lucius *et al.*, 1995; Mentlein and Roos, 1996; Mizutani *et al.*, 1993; Safavi and Hersh, 1995).

Aminopeptidase A truncates angiotensin II to brain angiotensin III, which exerts a tonic stimulatory action on the central control of blood pressure. Thus, central inhibitors of APA constitute putative central antihypertensive agents. APA is expressed in many tissues; among hematopoietic cells (B cell stages) it is lineage and differentiation stage specific (BP-1/6C3 antigen). With respect to angiotensin II catabolism endothelial cells are an important localization (Bausback *et al.*, 1988; Barrett *et al.*, 1998; Mentlein and Roos, 1996; Rozenfeld *et al.*, 2002).

Aminopeptidase P (the cell surface form is termed aminopeptidase P2 to distinguish it from the soluble form P1 that is coded by a different, related gene, see above) liberates N-terminal Arg from bradykinin; the following Pro-Pro residues may be further liberated by DPP IV (Orawski *et al.*, 1987). APP is anchored via a GPI anchor on the cell surface. The inactivation of bradykinin appears to be important in the lung, especially in rodents, where high APP activity is observed (Prechel *et al.*, 1995). Inactivation of neuropeptide Y appears to be of less importance (Mentlein *et al.*, 1993a; Orawski and Simmons, 1995). Interestingly, some ACE inhibitors also inhibit APP (Hooper *et al.*, 1992; Kim *et al.*, 2000); this effect may be additionally responsible for some side effects of ACE inhibitors. Membrane-bound APP is found in kidney and small intestine brush border membranes and endothelial and lymphoid cells (Barrett *et al.*, 1998). There is one report of (probable) human APP deficiency showing peptiduria of Xaa-Pro peptides, but with no other clinical symptoms (Blau *et al.*, 1988).

Pyroglutamyl-peptidase II is a strict substrate-specific enzyme: It liberates the terminal pyroglutamic acid from thyrotropin-releasing hormone (TRH); other pyroglutamyl peptides such as gonadoliberin or neurotensin are not attacked (Bauer, 1987, 1994). The enzyme is found in particular in the brain (neurons), anterior pituitary (mammotrops), retina, lung, and liver (Bauer *et al.*, 1990); also a soluble serum, obviously shedded form exists (Schmitmeier *et al.*, 2002). The extraordinarily high degree of substrate specificity and the restricted localization indicate that pyroglutamyl-peptidase II has a very specialized function as an inactivation enzyme for TRH as a neurotransmitter in the brain and as a hypothalamic releasing hormone in the circulation (Bauer 1994; Schomburg *et al.*, 1999). Dipeptidyl-peptidase IV and its physiological roles are discussed in detail below.

2. Carboxypeptidases and Dipeptidases

Carboxypeptidase M (CPM) cleaves the basic amino acids Arg or Lys from a variety of peptide substrates. Bradykinin, Arg-6-/Lys-6-enkephalins, and dynorphin A₁₋₁₃ appear to be natural substrates. It is a widely distributed enzyme and is anchored to the cell surface by a GPI anchor. The highest levels are found in lung, placenta, kidney, intestine, blood vessels, brain, and peripheral nerves. On hematopoietic cells CPM is found on discrete B lymphocyte developmental stages and on mature (mostly activated) T cells (de Saint-Vis *et al.*, 1995). Also, soluble forms exist in various body fluids. CPM is supposed to inactivate bradykinin partially: CPM-truncated bradykinin is inactive at the B2 receptor, but binds to the B1 receptor. Furthermore, CPM removal of the C-terminal Arg of human anaphylatoxin C5a abolishes its spasmogenic and histamine-releasing activity, whereas about 10% of the chemotactic activity is retained. Also, several growth factors (epidermal growth factor, hepatocyte growth factor, erythropoietin, brain-derived neurotrophic factors, vascular endothelial growth factor, oncostatin M, and others) are truncated by CPM, but the physiological importance remains to be established (Barrett *et al.*, 1998; Mentlein and Roos, 1996; Skidgel and Erdős, 1998).

Carboxypeptidase P liberates C-terminal amino acids from peptides with a penultimate Pro. So far, a role in the intestinal digestion of proline-containing peptides/fragments has been recognized, although some regulatory peptides (angiotensin II, enterostatin) have been also identified as substrates (Hedeager-Sørensen and Kenny, 1985; Mentlein and Roos, 1996; Yoshioka *et al.*, 1988).

Angiotensin-converting enzyme (ACE) liberates dipeptides from small peptides provided that the ultimate and penultimate residues are not Asp/Glu or Pro. The main physiological substrates are angiotensin I and bradykinin. However, the substrate specificity is relatively broad and enkephalins, dynorphins, neurotensin, and other are also cleaved. Furthermore, ACE also displays endopeptidase activities toward des-Arg-9-bradykinin, dynorphin, substance P, and gonadoliberin. ACE exists in two isoforms, a somatic form of 150–180 kDa in endothelial, epithelial, and neuronal cells, and a smaller isoform (90–110 kDa) in germinal cells. Furthermore, a soluble (shedded) form exists. Based on the conversion of angiotensin I to (active) angiotensin II by ACE, the control of blood pressure and salt metabolism is regarded as its main physiological role. Consequently, highly specific ACE inhibitors have been developed that are widely used as antihypertensive drugs. The main side effects of ACE inhibitors are attributed the prolongation of the lifetime/effects of bradykinin. However, ACE may also have another function as a regulatory peptidase, especially in the brain. For details on this well-investigated cell-surface peptidase and its physiological and

pharmacological importance the reader is referred to detailed reviews (Barrett *et al.*, 1998; Corvol *et al.*, 1995; Menard and Patchett, 2001; Skidgel *et al.*, 1987). In addition to the long known ACE, a further similar cell-surface enzyme has been recently discovered, *angiotensin-converting enzyme-2* (ACE2, ACEH), which also converts angiotensin I to angiotensin II, but not bradykinin. ACE2 is not inhibited by lisinopril or captopril. The organ- and cell-specific expression of ACE2 and its unique cleavage of key vasoactive peptides suggest an essential role for ACE2 in the local renin-angiotensin system of the heart and kidney (Danilczyk *et al.*, 2003; Turner *et al.*, 2002).

Membrane dipeptidase (MBD, MDB-1) is a GPI-anchored enzyme in various tissues, e.g., kidney and lung. It acts on dipeptides, peptidyl leukotrienes, and some β -lactam antibiotics. In the kidney it is implicated in the metabolism of glutathione (after removal of the glutamate residue by γ -glutamyl transpeptidase). In the lung it converts the peptidyl leukotriene D₄, a major component of the slow-reacting substances of anaphylaxis (broncho- and vasoconstrictors), to the inactive leukotriene E₄. Furthermore, some β -lactam antibiotics are cleaved and inactivated (Barrett *et al.*, 1998). Recently, additional members of this dipeptidase family have been identified (Habib *et al.*, 2003).

3. Endopeptidases

Neprilysin or *neutral endopeptidase* (NEP) preferentially cleaves peptide bonds with bulky, hydrophobic residues in the P₁ position as an endopeptidase. With some substrates it also displays a peptidyl-dipeptidase character (enkephalin, dynorphin). Peptides up to 40 residues are cleaved, although the efficiency of hydrolysis declines with peptide length. The principal substrates are enkephalins, atrial natriuretic peptides, substance P, and other tachykinins, but various other regulatory peptides (e.g., somatostatin, neurokinin, cholecystokinin-8, bradykinin, gastrin-releasing peptide, calcitonin, calcitonin gene-related peptide, and the chemotactic bacterial peptide fMet-Leu-Phe) are cleaved (Kenny, 1977; Kenny and Hooper, 1991). A recently discovered target is the amyloid β -peptide, whose accumulation causes Alzheimer's disease in the brain (Shirotani *et al.*, 2001). Endopeptidase is widely distributed in various tissues (e.g., kidney, intestinal and placental brush border membranes, immune cells, choroids plexus, some neurons in the brain, and Schwann cells). Thus, NEP inhibitors may have potential in the treatment of hypertension (already used in clinical trials), pain, and inflammation. For detailed information the reader is referred to special reviews (Barrett *et al.*, 1998, or the MERPOS database at <http://merops.sanger.ac.uk/>).

Endothelin-converting enzyme 1 is structurally related to NEP and is physiologically involved in the activation of endothelins, potent vasoconstrictors

generated from the intermediate proform big endothelin. Big endothelins are biologically inactive 38–41 amino acid residue intermediates that are generated from the endothelin peptide precursors of approximately 200 residues by prohormone processing enzymes (furines) in the secretory vesicles. Activation of the big endothelins to the active, 21-residue vasoconstrictors endothelin-1, -2, or -3 (ET-1 to -3) is accomplished by cleavage of the Trp-21-↓-Val-22 /Ile-22 bonds by ECE. ECE-1 has been shown to be located either at the cell surface or in the Golgi depending on the tissue. The related ECE-2 appears to be Golgi protein and, therefore, in a strict sense is not a cell-surface protease (see above). Consequently, these enzymes are potential drug targets for the treatment of hypertension ([Barrett *et al.*, 1998](#)).

B. Further Roles of Cell-Surface Peptidases (CSP)

1. CSP as Binding Proteins/Receptors

Cell-surface peptidases are rather large, highly glycosylated proteins. Compared with simple proteases such as trypsin with molecular masses between 20 and 30 kDa, huge portions of their peptide chains appear not to be necessary for enzymatic activity. Therefore, it is not surprising that the extracellular domain of some cell-surface peptidases fulfills other functions as binding protein or as a receptor. In fact, for certain cell-surface peptidases structure homologues without peptidase activity exist that appear to have binding functions.

DPP IV is an excellent example of a cell-surface peptidase with a dual function as an enzyme and a binding protein (see below): (human) DPP IV acts as a binding molecule for adenosine deaminase, an important enzyme for cellular and humoral immunity, as well as for extracellular matrix substances. Furthermore, DPP IV belongs to a gene family with proteins specialized in peptidase function (DPP IV) and binding functions: DPP6 and DPP10 have a transmembrane structure similar to DPP IV, are supposed to have arisen by gene duplication, but lack peptidase activity and appear to be binding proteins. Details are given below.

Specific expression of cell-surface peptidases might also contribute to the specialization of certain tissues. The specialization of the breast vasculature has been attributed to the expression of APP in this tissue ([Essler and Ruoslahti, 2002](#)). APP is able to bind certain synthetic peptide sequences that mimic structures of cancer cells responsible for metastasis into distinct tissues.

Viruses use cell-surface molecules for infection after endocytosis. Apart from receptors and carbohydrates, peptidases also might serve this function. In fact, APN acts as a receptor for the coronavirus 229E, which infects

epithelial cells of the respiratory tract and mediates human cytomegalovirus infection (responsible for Herpes) by enhancing its binding to the surface of infected cells (Delmas *et al.*, 1992).

In summary, the main function of cell-surface peptidases appears to be their enzymatic activity, but they may have additional binding activities. Structure homologues can have evolved that specialize in this function (or, less likely, vice versa, the cell surface may have evolved from cell-surface-binding proteins). Furthermore, as plasma membrane proteins they can function for binding/entry of viruses or homing of other cells/cancer cells.

2. CSP and Intracellular Signal Transduction

The intracellular sequence of cell-surface peptidases is normally very short (less than 20 amino acids) and contains no obvious motifs. Therefore, direct signal transduction effects without association with other functional molecules cannot be expected. However, in connection with other receptors or cell-membrane-associated enzymes signal transducing effects have been observed, in particular for APN, DPP IV, and γ -glutamyl transpeptidase (γ -GT). Induction of the signals may be triggered by inhibitors, substrates, or binding proteins.

Inhibition of either APN gene expression or of APN enzymatic activity has profound effects on proliferation and function in T cells (Lendeckel *et al.*, 2003). The most prominent changes observed after application of inhibitors such as actinonin or probestin include the activation of mitogen-activated protein kinases (MAPK) Erk 1/2 (extracellular-regulated kinases) or the Wnt pathway, a decrease in the production of proinflammatory cytokines (interleukin-2 and -12), and the induction of the immunosuppressive cytokine transforming growth factor- β (TGF- β). However, the precise mechanism, whether direct or indirect, remains speculative.

DPP IV is regarded as a costimulator of T cells (Kähne *et al.*, 1999). Association with antigens, binding proteins, especially adenosine deaminase and extracellular matrix proteins, or inhibitors induces cytokine production and proliferation. The protein tyrosine phosphatase CD45 as well as MAPK appears to be involved in this process (see below for details).

Since γ -GT is a key enzyme for the homeostasis of reduced glutathione (GSH), it is implicated in the regulation of the cellular redox state. Furthermore, in B cells γ -GT is associated with CD19 and members of the tetraspan 4 family including CD53, CD81, and CD82 (Nichols *et al.*, 1998). It remains unclear whether antigen activation or the modulation of oxidative signals could be responsible for the observed effects on intracellular signal transduction systems (Paolicchi *et al.*, 2002).

Taken together, signal transduction effects of cell-surface peptidases have been reported despite their short cytoplasmic domain (Antczak *et al.*, 2001a).

It appears, however, that these effects result mainly from their association with other cell-surface molecules.

3. CSP in Cell Invasion

Cell-surface peptidases might be implicated in cell invasion by their possible ability to adhere to extracellular matrix proteins, to degrade them, or to induce genes involved in this process. At present, there is some experimental evidence for such roles of cell-surface peptidases, especially for DPP IV and APN, but the exact mechanisms remain uncertain (Atherton *et al.*, 1992; Shingu *et al.*, 2003). DPP IV has been shown to bind to collagens I and III (see below). Furthermore, close structure homologues of DPP IV are devoid of enzymatic activity and appear to act only as binding proteins (see below). So far, the function of cell-surface peptidases (and their inactive homologues) in cell invasion and tissue integration is relatively unexplored, but is an interesting emerging field of research.

4. CSP in Diseases

Many investigations have been conducted on the aberrant expression of cell-surface peptidases in various diseases. To be distinguished is whether the activity of soluble forms in body fluids was measured, or whether the expression of membrane-bound forms was determined by activity measurements, enzyme/immunohistochemical stainings, or flow cytometric (FACS) analysis. For soluble activity, these studies are hampered by little knowledge of their shedding from the cell surface and its regulation. For the membrane-bound forms little is known about their induction by growth factors and their corresponding transcriptional elements, which are often tissue/cell-type specific. These aspects are covered by two recent reviews (Antczak *et al.*, 2001a,b).

Generally, high expression (in body fluids or at the cell surface) is correlated with hyperplasia of the tissue normally expressing the cell-surface peptidase, e.g., in cancer and chronic inflammatory diseases. Some cell-surface peptidases are also excellent markers for tissue differentiation, especially those expressed on hematopoietic cells (see cell-surface peptidases and CD nomenclature); therefore, they can be used to monitor this process or identify neoplastic transformations, in particular of leukemias and lymphomas. Also, cell activation can sometimes be detected by their elevated expression (see previous examples), although the precise regulation by growth factors/cytokines or a developmental program is less known. Today, there is also little knowledge of whether tissue-dependent regulation of their expression and consequently their peptidase activity influences their main physiological function, namely the inactivation of regulatory peptides.

5. Elucidation of the Physiological Functions by Knockout Animals

Although caution should be exercised in extrapolating results of animal/mice models to humans, targeted inactivation or inherited defects of the genes for cell-surface peptidases will provide important hints concerning their physiological functions (Table IV). Whereas in a few examples (ACE and ECE-1) abnormalities are directly evident, in most other examples investigated so far defects or physiological advantages are visible only under certain experimental conditions. This points to a nonredundant, intrinsic role only for certain surface peptidases, whereas most others are of importance in certain environmental or stressor situations (or are redundant genes). Nevertheless, NEP or DPP IV knockout mice/defective rats demonstrate that these enzymes function as regulatory peptidases not only *in vitro*, but that they are important regulators of neuropeptides and peptide hormones also *in vivo*. Interestingly, beside this main function they also appear to participate in more complicated diseases such as tumor metastasis or Alzheimer's disease.

Interestingly, human deficiencies or mutations of cell-surface peptidase genes are not well explored (there is a rare, not clear documented example for APP: Blau *et al.*, 1988). However, increasing information about genetic variations will certainly reveal their role as susceptibility or disease genes. This can be anticipated in particular for genes of cell-surface peptidases for which inhibitors are used as drugs.

C. Dipeptidyl Peptidase IV as an Example of the Diverse Functions of Cell-Surface Peptidases

Dipeptidyl-(amino)peptidases (DPP, EC 3.4.14) liberate dipeptides from the N-terminus of peptides. With the exception of DPP IV, they are lysosomal or cytosolic proteases. Two types of specificities can be distinguished: DPPs with relative broad specificity (DPP I, lysosomal Cys-protease; DPP III, cytosolic metalloprotease), but that are restricted by a proline in the second, the N-terminal penultimate position, and DPPs with complementary specificity (DPP II, DPP IV, and DPP8) liberating Xaa-Pro or Xaa-Ala dipeptides from the N-terminus. Generally, all DPPs act mainly on short or medium chain peptides (up to 80 residues) and less on larger proteins. Both types of DPPs could be regarded as merely digestive or as regulatory proteases. As digestive enzymes they are involved in the final degradation of peptides, either intra- or extracellularly. As regulatory enzymes they activate or inactivate bioactive peptides. Since these are extracellular communication molecules, only cell-surface dipeptidyl peptidase IV (DPP IV)/CD26 is of physiological importance.

In recent years, DPP IV has attracted considerable interest as a drug target, in particular for the treatment of type 2 diabetes. Therefore, this enzyme will be described in detail as an example of the importance of cell-surface peptidases (De Meester *et al.*, 1999; Gorrell *et al.*, 2001; Kähne *et al.*, 1999; Mentlein, 1988, 1999). Despite no surface oligopeptidases, brief information will be given on DPP IV-related molecules.

1. Molecular Properties

Human DPP IV is a dimeric 240-kDa glycoprotein composed of two 120-kDa subunits (Table V). The mRNA codes for a type II integral membrane protein of 766 amino acids (Misumi *et al.*, 1992) that is anchored in the plasma membrane by a 22 amino residue hydrophobic membrane-spanning domain (VLLG LLGAAALVTI ITVPVLL) preceded by a short, intracellular hydrophilic sequence (MKTPWK) at the N-terminus. The extracellular sequence contains nine potential N-linked glycosylation sites. Due to the presence of sialic acids in the carbohydrate structure, DPP IV has an acidic isoelectric point. The rat sequence exhibits about 85% identity to the human sequence, lacking one residue and one glycosylation site (Misumi *et al.*, 1992). The C-terminal regions (residues 625–752) of the human and rat sequences are completely conserved.

The crystal structure of DPP IV homodimers has been published recently (Engel *et al.*, 2003; Rasmussen *et al.*, 2003). Each subunit consists of two domains: an α/β -hydrolase domain and an eight-bladed β -propeller domain. Between these two domains, a large cavity of ~ 30 – 45 Å is found that is accessible via two openings. Substrates and products pass either through a funnel in the center of the propeller domain or through a much larger opening in the side between the domains.

The human DPP IV gene is located on the long arm of chromosome 2 (2q24.3) and spans approximately 82 kb. It contains 26 exons, ranging in size from 45 bp to 1.4 kb (Abbott *et al.*, 1994). The 5'-flanking domain contains neither a TATA box nor a CAAT box, but a 300-bp region extremely rich in C and G (72%) has potential binding sites for several transcription factors, such as NF κ B, AP2, or Sp1 (Table V, Abbott *et al.*, 1994; Böhm *et al.*, 1995). The DPP IV gene encodes two mRNAs sized about 4.2 and 2.8 kb that are expressed at different relative rates in individual tissues.

2. Localization

DPP IV has a wide tissue distribution; it is found on many epithelial and certain specialized mesodermal cells (Table V). It is often present at sites of physiological borders (e.g., the blood–brain barrier). Most important for its

TABLE V

Dipeptidyl Peptidase IV (DPP IV, DP4) and Fibroblast Activation Protein (FAP) α (FAP, Seprase)

| Abbreviations | DPP IV, DP4 | FAP |
|------------------------|--|---|
| EC number | 3.4.14.5 | 3.4.21.– |
| Alternative names | Dipeptidyl aminopeptidase IV Xaa-Pro-dipeptidylaminopeptidase Gly-Pro naphthylamidase Postproline dipeptidyl aminopeptidase IV CD26 Adenosine deaminase binding protein-2 Glycoprotein gp110 | Seprase |
| Genes | 2q24.3 (81.8 kb, 26 exons) | 2q24.2 (72.9 kb) |
| Transcription elements | SP-1, AP2, NF κ B, BRE, HNF-1, GAS | |
| mRNA | 4.2 and 2.8 kb (tissue dependent) | |
| Protein | 766 aa, glycosylated 120 kDa Native protein dimeric 240 kDa | 760 aa, glycosylated 97 kDa Native protein dimeric 170 kDa |
| Active site | <u>Ser</u> -. . . -Asp-. . . -His GWSYG | <u>Ser</u> -. . . -Asp-. . . -His GWSYG |
| Distribution | Endothelial cells Epithelial cells (intestine, pancreatic and bile duct, renal proximal tubular cells, spleen, sinus lining cells) Mature thymocytes Activated T cells Hepatocytes Specialized fibroblasts (myofibroblasts, synovial fibroblasts) | Reactive fibroblasts (development, tissue repair, cancer) Cultivated melanocytes Melanoma cells |
| Reaction catalyzed | Xaa-Pro/Ala- -Yaa. . ., Yaa \neq Pro | |
| Assay substrate | Gly-Pro- -NA (or AMC) | Gelatin, denatured collagen I, IV, also Gly-Pro-?-AMC |
| pH optimum | 7.8–8.0 | Neutral |

physiological functions (see below) is its location on endothelial cells of blood vessels, on activated T cells, on renal proximal tubular cells, and on intestinal epithelial cells (Fig. 2). Electron microscopy clearly shows its presence at the cell surface, but little is known about the dynamics of DPP IV from the plasma membrane to intracellular vesicles during endocytotic processes (Fig. 2C). It appears that DPP IV is associated with glycolipid- and cholesterol-rich membrane microdomains known as “rafts” rather than coated pits (Riemann *et al.*, 2001; Fig. 2C).

In addition to the membrane-bound form, soluble DPP IVs exist. These have no transmembrane domain: the human serum DPP IV lacks the first 39 residues (Durinx *et al.*, 2000; Iwaki-Egawa *et al.*, 1998) and the seminal

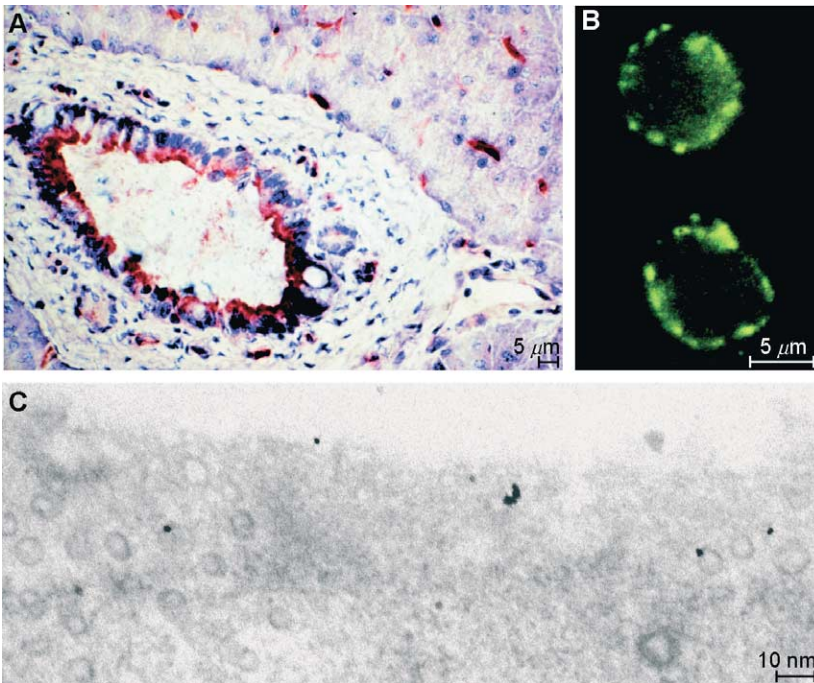


FIG. 2 Localization of DPP IV. (A) Histochemical staining of DPP IV activity with Gly-Pro-4-methoxy-2-naphthylamide and a diazonium salt on a fresh section of porcine pancreas. Epithelial cells of the pancreatic duct and endothelial cells of blood vessels are stained red; blue: nuclear counterstaining with hemalum. (B) Immunostaining of human T lymphocytes with rabbit antihuman DPP IV followed by a fluoresceine-labeled secondary antibody. The green immunofluorescence covers the cell surface. (C) Preembedding immunostaining of cultivated endothelial cells (human umbilical vein endothelial cells) with rabbit antihuman DPP IV followed by a 20-nm gold-labeled secondary antibody. Staining is found on the cell surface, in endosomal pits, and in vesicles (probably caveolae). A coated pit (at the left) is immunonegative.

plasma form lacks the first 29 or 32 residues (Durinx *et al.*, 2000; Ohkubo *et al.*, 1994). These soluble forms are considered to be produced by proteolytic shedding from the cell surface of endothelial or epithelial cells. However, the proteases responsible for their release and the conditions activating this process have not yet been identified.

3. Catalytic Properties

DPP IV was discovered as an enzyme liberating naphthylamine from Gly-Pro-2-naphthylamide and was initially termed glycylproline naphthylamidase (Hopsu-Havu and Glenner, 1966). In fact, mammalian DPP IV exhibits restricted substrate specificity (Fig. 3).

In the P₁ position proline and alanine are accepted almost exclusively. Peptides or chromogenic substrates with Pro are better hydrolyzed than corresponding ones with Ala. However, important natural peptide substrates have both, penultimate Pro or Ala (Table VI). Apart from these, few peptides with other small P₁ residues (e.g., Ser, Val, Gly, Leu) are cleaved, although with considerably lower rates, e.g., glucagon (Hinke *et al.*, 2002; Martin *et al.*, 1993; Pospisilik *et al.*, 2001), analogues of growth hormone-releasing hormone (GRH) (Bongers *et al.*, 1992), and macrophage-derived chemokine (MDC) from which Gly-Pro and Tyr-Gly are sequentially released (Proost *et al.*, 1999).

In the P₂ position any unsubstituted proteinogenic amino acid is accepted (including proline). Substrates with hydrophobic or basic residues are better

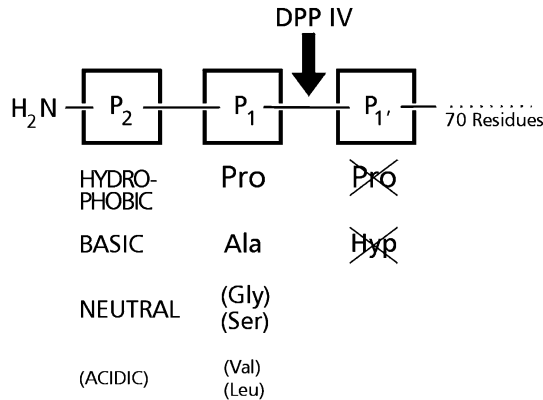


FIG. 3 Schematic representation of substrates cleaved by DPP IV. Dipeptides are liberated from the N-terminus of peptides with Pro or Ala in the P₁ position. Certain peptides with other small amino acids in the P₁ position are cleaved with low rates. In the P₂ position bulky, hydrophobic, or basic amino acids with an obligate free amino group are preferred. Peptides with Pro or Hyp in the P₁' position are resistant to DPP IV.

hydrolyzed than those with acidic ones. The peptide bond between the P₁ and P₂ residues must be in the *trans* position. Peptides with Pro or Hyp in the P_{1'} position are not cleaved, e.g., bradykinin (Arg-Pro-Pro-...). The chain length of the peptide substrates has not been systematically investigated. Peptides up to 70 residues are degraded; sequestration of larger proteins may depend on their tertiary structures (Nausch *et al.*, 1990). This restriction can be explained by the three-dimensional structure of DPP IV (Rasmussen *et al.*, 2003). Optimal cleavage rates are generally observed at pH values between 7.5 and 8.5.

Apart from this action as a strict exopeptidase, endopeptidase activities on collagen or other extracellular matrix proteins have been postulated occasionally for DPP IV (Bermppohl *et al.*, 1998). At present it can, however, not be excluded that these endopeptidase activities result from proteases closely related to or associated with DPP IV, e.g., fibroblast activation protein α (seprase) that can form heterodimers with DPP IV.

DPP IV belongs to the serine class of proteases, clan SC, family S9 (compare Barrett *et al.*, 1998, or <http://merops.sanger.ac.uk/>). The catalytic triad, Ser-630, Asp-708, and His-740, is invertedly arranged as compared to the trypsin-like serine proteases (His-Asp-Ser); therefore, DPP IV is classified as a nonclassical serine protease. The active site sequence **GWSYG** around the catalytic serine residue (David *et al.*, 1993; Ogata *et al.*, 1992) corresponds to the consensus sequence **GXSXG** of serine proteases and is found in one copy at positions 628–632 (human sequence). A single substitution in this sequence in a substrain of Fisher-344 rats (G to R; active site **GWSYR**) results in loss of enzymatic activity. Due to this deficiency for active DPP IV, this Fisher-344 rat strain has become a valuable model to study physiological functions (Fujiwara *et al.*, 1992; Watanabe *et al.*, 1987).

In accordance with its catalytic structure, DPP IV is inhibited more or less powerfully by serine enzyme inhibitors that covalently modify their active site. Diisopropylfluorophosphate is a good inhibitor, whereas phenylmethylsulfonylfluoride is relatively ineffective (McDonald and Barrett, 1986; Püschel *et al.*, 1982). Metal ions such as Pb²⁺, Hg²⁺, and Zn²⁺ are strongly inhibitory. Inhibitors of other classes of proteases or inhibitors of aminopeptidases (e.g., bestatin) are ineffective.

Specific DPP IV inhibitors acting at low concentrations are of special interest for physiological investigations and for potential clinical applications. The microbial peptides diprotin A (Ile-Pro-Ile) and diprotin B (Val-Pro-Leu) are competitive substrates that are slowly hydrolyzed and act as selective competitive inhibitors for DPP IV at micromolar concentrations (Rahfeld *et al.*, 1991). In addition, several types of substrate analogues have been synthesized that are powerful and specific inhibitors at micromolar to picomolar concentrations, e.g., proline boronic acid dipeptide inhibitors (reversible), dipeptide phosphonates (irreversible), dipeptide 2-cyanopyrrolidides,

TABLE VI
Regulatory Peptides as Substrates for Dipeptidyl Peptidase IV

| Peptide | N-terminus | aa | Significance | References |
|---------------------|-----------------------|-------|---|---|
| <i>Inactivation</i> | | | | |
| Neuropeptides | | | | |
| Endomorphin-2 | YP-FF-NH ₂ | 4 | Inactivation (<i>in vivo</i>) | Shane <i>et al.</i> , 1999 |
| Hormones | | | | |
| GRH | YA-D... | 29/44 | Inactivation (<i>in vivo, in vitro</i>) | Kubiak <i>et al.</i> , 1989, 1998; Mentlein <i>et al.</i> , 1993b |
| GLP-1 | HA-E... | 30 | Inactivation (<i>in vivo, in vitro</i>) | Deacon <i>et al.</i> , 1995; Kiefer <i>et al.</i> , 1995; Mentlein <i>et al.</i> , 1993b |
| GLP-2 | HA-E... | 34 | Inactivation (<i>in vivo, in vitro</i>) | Drucker <i>et al.</i> , 1997a |
| GIP | YA-E... | 42 | Inactivation (<i>in vivo, in vitro</i>) | Deacon <i>et al.</i> , 2001; Mentlein <i>et al.</i> , 1993b |
| Glucagon | HS-QG-T... | 29 | Inactivation (<i>in vitro</i>) | Hinke <i>et al.</i> , 2000; Popsilik <i>et al.</i> , 2001 |
| Chemokines | | | | |
| SDF-1 (CXCL12) | KP-V... | 68/72 | Inactivation (<i>in vitro</i>) | Proost <i>et al.</i> , 1998; Shioda <i>et al.</i> , 1998 |
| MDC (CCL22) | GP-YG-A... | 69 | Inactivation (<i>in vitro</i>) | Proost <i>et al.</i> , 1999 |
| I-TAC (CXCL11) | FP-M... | 73 | Inactivation (<i>in vitro</i>) | Ludwig <i>et al.</i> , 2002; Proost <i>et al.</i> , 2001 |
| IP-10 (CXCL10) | VP-L... | 77 | Inactivation (<i>in vitro</i>) | Proost <i>et al.</i> , 2001 |
| Mig (CXCL9) | TP-V... | 103 | Inactivation (<i>in vitro</i>) | Proost <i>et al.</i> , 2001 |
| Eotaxin (CCL11) | GP-A... | 74 | Inactivation (<i>in vitro</i>) | Oravec <i>et al.</i> , 1997 |
| LD78β (CCL3) | AP-L... | 69 | Inactivation (<i>in vitro</i>) | Proost <i>et al.</i> , 2000 |

Partial inactivation

Neuropeptides

| | | | | |
|----------------|------------|----|---|--|
| Neuropeptide Y | YP-S... | 36 | At Y1 receptors <i>in vitro</i> | Mentlein et al., 1993a |
| Substance P | RP-KP-Q... | 11 | Lower binding NK ₁ receptor Neurogenic inflammation | Heymann and Mentlein, 1978 Grouzmann et al., 2002 |

Hormones

| | | | | |
|------------|---------|----|---------------------------------|--|
| Peptide YY | YP-I... | 36 | At Y1 receptors <i>in vitro</i> | Mentlein et al., 1993a |
|------------|---------|----|---------------------------------|--|

Chemokines

| | | | | |
|---------------|---------|----|----------------------------------|-------------------------------------|
| RANTES (CCL5) | SP-Y... | 68 | At CCR1 and CCR3 <i>in vitro</i> | Oravec et al., 1997 |
|---------------|---------|----|----------------------------------|-------------------------------------|

Cleavage with no or unknown physiological effects (examples)

| | | | | |
|---|------------|----|--------------------|-------------------------------------|
| Enterostatin | VP-DP-R | 5 | Inactivation? | Bouras et al., 1995 |
| Corticotrophin-like intermediate lobe peptide | RP-V... | 22 | Probably no effect | Nausch et al., 1990 |
| Gastrin-releasing peptide | VP-LP-A... | 27 | Probably no effect | Nausch et al., 1990 |
| GCP-2 (CXCL6) | GP-V... | 77 | No effect | Proost et al., 1998 |

or aminoacyl-pyrrolidides (both reversible; Villhauer *et al.*, 2001). Natural peptides with an N-terminal Xaa-Xaa-Pro sequence are more or less strong inhibitors of DPP IV, e.g., peptide YY(3–36) or the Tat protein of the human immunodeficiency virus type-1 (HIV-1) (Wrenger *et al.*, 1996).

4. Physiological Substrates and the Importance of DPP IV Cleavage

Mammalian DPP IV cleaves a variety of regulatory peptides—neuropeptides, peptide hormones, and chemokines (Table VI). This N-terminal truncation can inactivate, partially inactivate (to certain receptor subtypes/effects), or have no (or an unknown) effect on the biological activity of the peptide. Regulatory peptides with an Xaa-Pro-Pro ... sequence, e.g., bradykinin (RPP... , 9 residues) and longer peptides, e.g., interleukin-2 (APT... , 133 residues) or interleukin-1 β (APV... , 153 residues), are not cleaved (Mentlein, 1999). Activation of peptide precursors by DPP IV cleavage (or DPP IV-like enzymes) has only been observed in lower vertebrates and invertebrates, e.g., processing of the prohormone for caerulein in the frog *Xenopus laevis*, of an antifreeze protein in the winter flounder, proceropins (antibacterial peptides) in moth, promelittin (bee venom) in the honeybee, or preprohormones in sea anemones (Martensen *et al.*, 1998).

The relative degradation rates of the different peptides can vary considerably, and some of the reported substrates are cleaved only after prolonged incubation times *in vitro* (Lambeir *et al.*, 2001b; Mentlein, 1999, Zhu *et al.*, 2003). Therefore, the physiological significance of DPP IV truncation remains questionable for some substrates, e.g., glucagon and most chemokines. However, a pivotal role of DPP IV in the final or partial inactivation of some peptide hormones (GLP-1, GLP-2, GIP, and peptide YY) and neuropeptides (neuropeptide Y, substance P, PACAP-38, and endomorphin-2) *in vivo* is evident. For chemokines a function of DPP IV truncation *in vivo* has not yet been proven, but may be anticipated for some (SDF-1, MDC, and I-TAC), at least under certain physiological conditions.

Circulating peptide hormones are exposed to large quantities of DPP IV on the surface of endothelial cells. In comparison, soluble serum DPP IV or CD26 on leukocytes has less total activity (Mentlein, 1999), but contributes further to hormone degradation. The most important physiological and pharmacological DPP IV substrates to date are the incretin hormones GLP-1 and GIP. They belong to a family of 27–44 residue peptides, the glucagon peptide family. Those with a penultimate Ala, namely GRH, GLP-1, GLP-2, GIP, and PHM, are good DPP IV substrates (Lambeir *et al.*, 2002; Mentlein *et al.*, 1993b); those with a penultimate Ser, namely glucagon, secretin, VIP, and PACAP-27, are relatively poor substrates (Hinke *et al.*, 2000; Lambeir *et al.*, 2001a) and will not be discussed here.

Glucagon-like peptide-1 [GLP-1; formerly GLP-1(7–36)NH₂] and glucagon-like peptide-2 (GLP-2) are, respectively, 30- and 33-residue gastrointestinal peptide hormones released postprandially from enteroglucagon cells (L cells) in the small and large intestine, and are generated by an alternative posttranslational processing of proglucagon (positions 78–108 and 126–159 of the human proglucagon precursor). GLP-1 stimulates insulin secretion *in vitro* and *in vivo*. It plays an important role in the regulation of postprandial insulin release and is the most important incretin (insulinotropic hormone) known so far (Fig. 4). In patients with type 2 diabetes, exogenous GLP-1 lowers postprandial plasma glucose excursions by stimulating insulin release and by inhibiting glucagon secretion. Since the biological half-life of GLP-1 is very short, the use of exogenous GLP-1 or prolongation of its action is considered as a new possibility in the treatment of patients with type 2 diabetes. GLP-1 is also produced in the brain, and intracerebroventricular GLP-1 in rodents is a potent inhibitor of food and water intake.

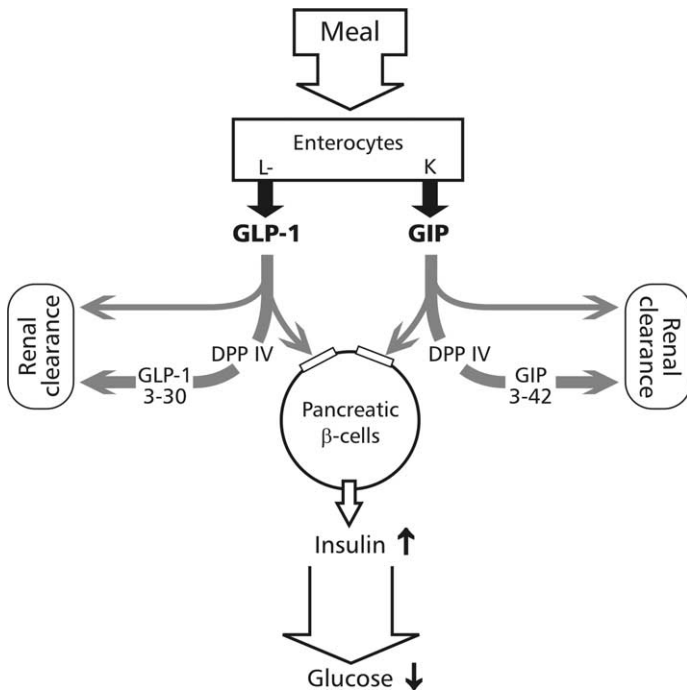


FIG. 4 Schematic drawing of the role for DPP IV in the inactivation of incretins. GLP-1 and GIP are released postprandially from intestinal L or K cells, transported in the blood, and stimulate insulin secretion from pancreatic β -cells. DPP IV at the surface of endothelial cells or soluble in the blood degrades both peptides at the N-terminus resulting in a rapid loss of hormonal activity.

GLP-2 displays an intestinal growth factor activity in rodents, raising the possibility that GLP-2 may be therapeutically useful for enhancement of mucosal regeneration in patients with intestinal diseases (Drucker, 1998, 2003a). Understanding the inactivation of GLP-1 and GLP-2 is therefore of potential therapeutic interest.

Several studies *in vitro* and *in vivo* have shown that GLP-1 is primarily degraded at the N-terminus by the action of DPP IV resulting in biologically inactive des(His-Ala)-GLP-1 (Deacon *et al.*, 1995a,b, 1998a,b; Kieffer *et al.*, 1995; Mentlein *et al.*, 1993b). After subcutaneous or intravenous administration of GLP-1 in healthy or type 2 diabetic humans more than 75% was recovered from the plasma as des(His-Ala)-GLP-1 (Deacon *et al.*, 1995b). After intravenous injection of ¹²⁵I-radiolabeled GLP-1 at picomolar concentrations into rats more than 50% was metabolized to des(His-Ala)-GLP-1 within 2 mins, but not in DPP IV-negative rats (Kieffer *et al.*, 1995). This conversion could be blocked *in vitro* and *in vivo* by specific DPP IV inhibitors such as diprotin a or Lys- or Val-pyrrolidide. Pharmacological inhibition of DPP IV with valine-pyrrolidide in anesthetized pigs, at a dose that reduced plasma DPP IV activity by more than 90%, increased the amount of intact GLP-1 both in the basal state and after infusion (Deacon *et al.*, 1995b). Moreover, inhibition of DPP IV by Ile-thiazolidide in Zucker rats potentiated the insulinotropic effect of GLP-1 after intravenous infusion alone or with glucose (Pederson *et al.*, 1998). Furthermore, glucose tolerance and insulin secretion are enhanced in DPP IV knockout mice (Marguet *et al.*, 2000) and in DPP IV activity-deficient Fischer 344 rats (Nagakura *et al.*, 2001) in comparison to normal mice or Fischer rats. However, inhibition of DPP IV in these normal animals showed similar effects. Meanwhile, improved glucose tolerance under DPP IV inhibition has also been proven in humans (Ahrén *et al.*, 2002). Therefore, inhibition of DPP IV is regarded as a validated target for treatment of type 2 diabetes.

Like GLP-1, GLP-2 is also easily degraded to des(His-Ala)-GLP-2 by purified human DPP IV or with serum containing DPP IV activity (Drucker *et al.*, 1997a) as well as in rats and in humans *in vivo* (Hartmann *et al.*, 2000; Tavares *et al.*, 2000). When injected into normal rats, GLP-2 induces an increase in intestinal villus height and has a small trophic effect on small bowel weight. Administration of GLP-2 into DPP IV-deficient rats markedly increased the bioactivity of rat GLP-2 resulting in a significant increase in small bowel weight (Drucker *et al.*, 1997a). Again, DPP IV-resistant GLP-2 analogues show higher effects than GLP-2 in wild-type animals, e.g., a statistically significant increase in small bowel mass was produced by the analogue *r*[Gly(2)]-GLP-2 in rats and by the human analogue *h*[Gly(2)]-GLP-2 in mice (Drucker *et al.*, 1997a,b). These results show

that inactivation of GLP-2 by DPP IV is of critical importance for its growth factor properties in the intestine and that DPP IV-resistant analogues may be useful therapeutics to improve mucosal regeneration.

Gastric inhibitory polypeptide or glucose-dependent insulinotropic polypeptide (*GIP*), a 42-amino acid polypeptide, is secreted by duodenal K cells after ingestion of fat and glucose. It inhibits the secretion of gastric acid and stimulates insulin release from pancreatic β cells in the presence of elevated glucose levels. Thus, in addition to GLP-1, *GIP* also contributes to incretin activity of postprandial serum (Fig. 4), more in rodents and less in humans (Meier *et al.*, 2002). As the other members of the GRH-glucagon family with a penultimate Ala, *GIP* is also rapidly degraded to *GIP*(3–42) by purified human DPP IV or serum with DPP IV activity *in vitro* (Mentlein *et al.*, 1993b) and *in vivo* (Deacon *et al.*, 2000). This truncation of *GIP* in serum could be successfully inhibited by coincubation with specific DPP IV inhibitors *in vitro* and *in vivo* (Deacon *et al.*, 2001; Kieffer *et al.*, 1995; Mentlein *et al.*, 1993b). *GIP*(3–42) was also detected after purification of *GIP* from pig intestine (Jörnvall *et al.*, 1981), and later it was shown that it lacked any biological activity and is even a weak *GIP* receptor antagonist *in vivo* (Gault *et al.*, 2002; Schmidt *et al.*, 1987). As reported for GLP-1, *GIP* was also rapidly degraded *in vivo* to the truncated form (50% after 2 min) after injection of radioiodinated *GIP* into wild-type rats, but not in DPP IV-deficient ones (Kieffer *et al.*, 1995). In accordance, after oral administration of a DPP IV inhibitor the half-life of *GIP* was prolonged in wild-type rats or pigs (Deacon *et al.*, 2001; Kieffer *et al.*, 1995) or DPP IV-resistant analogues are more potent in normal or obese rats (Hinke *et al.*, 2002). Thus, as for GLP-1 and GLP-2, DPP IV truncation is also the main degradation route for *GIP in vivo*.

Growth hormone-releasing hormone (*GRH*) is a 44-residue peptide of which biologically active, C-terminal truncated fragments are known. It is released from the hypothalamus and stimulates the release and the synthesis of growth hormone from and in the pituitary. In human plasma *GRH*(1–44)-NH₂ (or fragments) is rapidly metabolized to *GRH*(3–44)-NH₂; further minor degradation products derive from endoproteolytic cleavage of the Arg(11)-Lys(12) or Lys(12)-Val(13) bonds (Frohman *et al.*, 1989). All these proteolytic fragments are biologically inactive. By use of specific inhibitors, DPP IV and a trypsin-like endoprotease were identified as the proteases responsible for this processing. Following this initial report, DPP IV-mediated proteolysis was established as a major route of *GRH* degradation and inactivation in rat (Boulanger *et al.*, 1992), porcine, and bovine plasma *in vitro* and in cattle *in vivo* (Kubiak and Friedman, 1998; Kubiak *et al.*, 1989). Again, DPP IV-resistant peptide analogues of *GRH*(1–29) showed similar biological activity to induce growth hormone release *in vitro*

(cultivated pituitary cells), but higher potency to increase serum growth hormone levels *in vivo* up to 60 min after injection into cattle due to longer stability *in vivo* (Kubiak and Friedman, 1998).

Peptide YY (PYY) is produced by endocrine cells of the small intestine and the colon and is released after a meal as a peptide hormone circulating in the blood that inhibits several gastrointestinal functions, e.g., gastric acid release. The amino terminal sequence Tyr-Pro is conserved in neuropeptide Y and PYY among all vertebrate species and both appear to be the best peptide substrates for DPP IV. Although PYY is rapidly cleaved by human serum and purified DPP IV *in vitro* (Mentlein *et al.*, 1993a) to PYY(3–36) and this fragmentation product is the major molecular form of PYY extracted from human or rabbit intestine (Eberlein *et al.*, 1989) or circulating in the blood (Grandt *et al.*, 1994), the physiological consequences have not been explored.

Among the *neuropeptides* degraded by DPP IV (endomorphin-2, substance P, neuropeptide Y; Table VI), neuropeptide Y (NPY) is probably the most interesting substrate. Together with PYY and pancreatic polypeptide (PP), it belongs to a peptide family of 36-residue peptides with high homology in primary and tertiary structure. NPY is an abundant neuropeptide in the central and peripheral nervous system; it is involved in the control of feeding, energy homeostasis, and blood pressure. As with PYY, NPY is also rapidly degraded by DPP IV *in vitro* to des(Tyr-Pro)NPY (Mentlein *et al.*, 1993a), and this truncated form has been identified as a considerable part (35%) of the total NPY extracted from porcine brain (Grandt *et al.*, 1996) and in the human cerebrospinal fluid (Nilsson *et al.*, 1998). Full-length NPY, PYY, and PP bind with different affinities to at least five receptor subtypes (Y1 and Y2—NPY and PYY, Y3—NPY > PYY, Y4—PP, and Y5—all three), but N-terminally truncated NPY (and PYY) lacks affinity to the Y1 receptor (Gerald *et al.*, 1996), which is located in the cerebral cortex and on vascular smooth muscle cells where it promotes vasoconstriction and cell proliferation. Due to the receptor subtype-specific regulation of NPY activity, DPP IV has already been termed an “NPY-converting enzyme.” In this respect, DPP IV appears to be involved in the regulation of vascular smooth muscle contraction, angiogenesis (Zukowska-Grojec *et al.*, 1998), and, in particular, endothelial cell migration (Gherssi *et al.*, 2001) and edema (Dimitrijevic *et al.*, 2002).

Substance P is a widespread neuropeptide that acts, e.g., as a transmitter of sensory information including noxious stimuli, as a potent contractor of smooth muscles, and as an immunoregulator. Three different G-protein-coupled receptor subtypes, NK₁, NK₂, and NK₃, are known that are also stimulated by neurokinins A and B, which share a common C-terminal pentapeptide sequence (Phe-Xaa-Gly-Leu-Met-NH₂) with substance P. Therefore, the sequential liberation of the dipeptides Arg-Pro and Lys-Pro

from the N-terminus of the undecapeptide substance P (Heymann and Mentlein, 1978; Kato *et al.*, 1978) does not abolish its biological activity at the tachykinin receptors. However, the fragment substance P-(5–11) has a lower binding activity for the preferred substance P receptor, the NK₁ receptor (Mussap *et al.*, 1993). Only effects for which the first four residues in substance P are essential, e.g., the degranulation of mast cells (probably non-receptor-mediated by perturbation of the membrane), may be completely abolished by DPP IV cleavage. However, substance P truncated by DPP IV can be further attacked by aminopeptidase N, which is an important degradation pathway in human plasma, at the vascular endothelium, at fibroblasts, and muscle myocytes (Wang *et al.*, 1991, 1994). This may explain the potentiation of neurogenic inflammation and enhanced vasodilation by DPP IV inhibitors in nasal mucosa (Grouzmann *et al.*, 2002).

Endomorphin-1 (YPWF-NH₂) and *-2* (YPPF-NH₂) are endogenous opioid peptides with high affinity at μ opioid receptors that produce potent analgesia in mice. DPP IV liberated the terminal Tyr-Pro with high activity from endomorphin-2, whereas an analogue with D-Pro in the penultimate position was resistant. This analogue was more potent and longer lasting to induce analgesia *in vivo*; moreover the DPP IV inhibitor Ala-pyrrolidonyl-2-nitrile itself elicited analgesia and potentiated the actions of endomorphin-2 (Shane *et al.*, 1999). These results suggest a role for DPP IV in endomorphin inactivation *in vivo*.

Among the *chemokines* cleaved by DPP IV (CCL3, CCL5, CCL11, CCL22, CXCL9, CXCL10, CXCL11, and CXCL12, Table VI), SDF-1 (CXCL12), I-TAC (CXCL11), and MDC (CCL22) appear to be most important, since they are cleaved with the highest rates (Lambeir *et al.*, 2001b). Chemokines constitute a large group of small (8–10 kDa) secreted proteins that act as cell-type-selective chemoattractants. They can be divided into four subfamilies, based on structural, functional, and genetic criteria. The two most important subfamilies are the CXC and CC chemokines (α - and β -family), which differ in the spacing of the first two cysteine residues that form disulfide links with two other cysteines. In CXC chemokines these first cysteine residues are separated by one amino acid; in CC chemokines they are directly neighbored. Generally, DPP IV cleavage of chemokines abolishes their chemotactic activity. In some cases the truncated forms act as weak antagonists. However, up to now no experimental evidence has been given on the importance of this process *in vivo*. DPP IV inhibitors often show an antiinflammatory effect (Steinbrecher *et al.*, 2001), not a potentiation of the inflammatory response as expected for prolongation of chemokine action. In accordance, DPP IV inhibitors did not enhance the chemotaxis of I-TAC (CXCL11) on activated human T cells *in vitro* that express both DPP IV/CD26 and the chemokine receptor CXCR3 (Mentlein *et al.*, 2003). However,

different results have been found for the chemotactic effect of SDF-1 α (CXCL12) on human cord blood CD34⁺ progenitor cells that also coexpress DPP IV/CD26 and the receptor CXCR4 (Christopherson *et al.*, 2002).

In conclusion, for several circulating hormones (GLP-1, GLP-2, GIP, GRH, and PYY), neuropeptides (NPY, substance P, and endomorphin), and chemokines (SDF-1 α , others) the role of DPP IV in their complete or receptor subtype-specific inactivation has been well documented. Pharmacologically most important is the action on GLP-1.

5. Pharmacological Importance of DPP IV

Despite its multiple functions in the degradation of several regulatory peptides and the invasiveness of tumors, DPP IV is at present mainly considered a new, validated target for treatment of type 2 diabetes (Augustyns *et al.*, 2003; Drucker, 2003b; Holst, 2002; Holst and Deacon, 1998). Prolongation of GLP-1 action by inhibition of its degradation or application of DPP IV-resistant GLP-1 analogues has been shown to enhance insulin secretion and glucose tolerance *in vivo*. GLP-1 analogues modified at the N-terminal His(1), Ala(2), and Glu(3) by the corresponding D- or different amino acids, e.g., D-Ala(2)-GLP-1, have longer half-lives and consequently higher insulinotropic potencies *in vitro* and *in vivo* (Burcelin *et al.*, 1999; Deacon *et al.*, 1998b; Siegel *et al.*, 1999a,b). Though this modification is a rather specific approach for type 2 diabetes with low side effects, it has the drawback that GLP-1 peptide analogues cannot be given orally, in contrast to small DPP IV inhibitors (Augustyns *et al.*, 2003), which have more potential side effects. However, recent clinical studies with an orally active DPP IV inhibitor in type 2 diabetic patients show a considerably better insulin tolerance and improved HBA_{1c} levels after 4 weeks of treatment (Ahrén *et al.*, 2002). Side effects were surprisingly low (partly nasopharyngitis, pruritus, and transient nephritic syndrome). Thus, DPP IV inhibitors appear to be promising drugs for treatment of some stages of type 2 diabetes.

6. DPP IV as a Binding Protein

Apart from its function as a regulatory protease, other important roles for DPP IV have been reported. Intestinal and renal DPP IV is involved in the final digestion of proline-containing oligopeptides and in the absorption of their fragments (Brandesch *et al.*, 1995). DPP IV also acts as a costimulatory and a binding protein.

DPP IV has been shown to be identical to the cell-surface marker CD26 (thymocyte-activating molecule THAM is the mouse homologue; 2B9 antigen) that is expressed on a fraction of resting T cells at low density

but strongly up-regulated following T cell activation (Dang and Morimoto, 2002; Gorrell *et al.*, 2001; Morimoto and Schlossman, 1998; Reinhold *et al.*, 2002; von Bonin *et al.*, 1998). CD26/DPP IV can deliver a potent costimulatory activation signal into T cells despite its small cytoplasmic region of only six amino acids. Signaling is dependent on its interaction with other molecules. It associates, presumably through its extracellular domain, with CD45, a protein tyrosine phosphatase, and with adenosine deaminase (ADA, EC 3.5.4.4), an enzyme involved in the salvage of purine nucleosides, which catalyzes the deamination of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine and whose deficiency causes severe combined immunodeficiency disease (SCID) in humans (DPP IV = adenosine deaminase complexing protein = ADAbp). Moreover, CD26 signaling is dependent on the expression of the T cell receptor (TCR) complex.

For tumor cells, endothelial cells, and trophoblasts, DPP IV expression is associated with a lower invasive or metastatic potential (Cheng *et al.*, 1998; Kajiyama *et al.*, 2002; Sato *et al.*, 2002; Shingu *et al.*, 2003; Wesley *et al.*, 1999). It could be that this effect is attributable to interaction with extracellular matrix substances, e.g., collagens I and III to which DPP IV binds via its cysteine-rich domain, not by its catalytic site, or fibronectin (Löster *et al.*, 1995). The dual function of DPP IV as a protease and a binding/stimulatory molecule is sustained by structural homologous proteins for which either one or the other function has been reported (see below).

7. DPP IV-Related Proteins

In the past years several DPP IV activity and/or structure homologues (DASH proteins) have been discovered (Sedo and Malik, 2001). Some of them appear to be rediscovered proteins with new names.

The following activity homologues also cleave chromogenic/fluorogenic DPP IV substrates such as Gly-Pro-4-nitroaniline, but are soluble enzymes.

Dipeptidyl peptidase II (DPP II, EC 3.4.14.2) appears to be identical (Leiting *et al.*, 2003) to quiescent cell proline peptidase (QPP) and dipeptidyl peptidase 7 (DPP 7/DPP VII). DPP II is a soluble lysosomal protein of 110 kDa consisting of two 53-kDa subunits and cleaves at acidic pH only short Xaa-Pro/Ala peptides (Mentlein and Struckhoff, 1989).

Dipeptidyl peptidase 8 (DPP8) is a cytosolic 82-kDa protein active at neutral pH with DPP IV activity (Abbott *et al.*, 2000). It might be identical to dipeptidyl peptidase IV- β .

Dipeptidyl peptidase 9 (DPP9) is related to DPP 8, a 98-kDa cytosolic protein with DPP IV activity (Olsen and Wagtmann, 2002).

The following structure homologues are cell-surface proteins, but they either lack enzymatic activity or exhibit a different spectrum. *Attractin*

(mahogany homolog, DPPT-L) is a 175-kDa type I transmembrane protein from which four secreted isoforms are produced by alternative splicing (Gunn *et al.*, 1999). It is expressed and secreted by activated T cells and at low levels by other tissues. Attractin has probably low DPP IV activity, but whether it also cleaves the same peptides has not been thoroughly investigated. The protein is supposed to be involved in initial immune cell clustering during the inflammatory response and may regulate the chemotactic activity of chemokines. Furthermore, it may play a role in melanocortin signaling pathways that regulate energy homeostasis and hair color.

Fibroblast activation protein α (FAP, seprase, 3.4.21-) is a type II transmembrane protein of 97-kDa subunits that has about 52% homology (human sequences) to DPP IV but acts mainly as an endopeptidase on extracellular matrix protein (Table V; Barrett *et al.*, 1998; Goldstein *et al.*, 1997; Scanlan *et al.*, 1994). In normal tissues, FAP is found only in fetal mesenchymal cells and a subset of glucagon-producing cells (A cells) in pancreatic islets. However, activated fibroblasts, either cultivated, in wounds or in tumors, cultivated melanocytes, and sarcoma cells express FAP. The biological function has not been clarified, but it is suspected that FAP is involved in wound healing. Due to its selective occurrence, FAP is a good target for tumor immunodetection and destructive tumor therapy. DPP IV and FAP reside on a neighbor gene locus (Table V) and may therefore originate by gene duplication. They can form homodimers, but usually do not occur together.

Glutamate carboxypeptidase II (GCP II, EC 3.4.17.21) or *N-acetylated α -linked acidic dipeptidase* (NAALADase I, also folate hydrolase, pteroyl-poly- γ -glutamate carboxypeptidase) is a type II transmembrane protein of 750 amino acids and about 84 kDa (Barrett *et al.*, 1998). It releases unsubstituted, C-terminal glutamyl residues from Ac-Asp-Glu or pteroyl- γ ; in particular it hydrolyzes γ -peptide bonds in Ac-Asp-Glu, Asp-Glu, and Glu-Glu, but also γ -glutamyl bonds in γ -Glu-Glu and folylpoly- γ -glutamates. According to more recent investigations it has no DPP IV activity (Barinka *et al.*, 2002). The enzyme requires zinc (2 per subunit), is activated by cobalt, and is inhibited by quisqualic acid, 2-(phosphonomethyl) pentanedioic acid (PMPA), and EDTA. Primary sources are prostate epithelium, as well as the small intestine (specifically in jejunum brush border membranes), brain (ventral striatum and brain stem), kidney, liver, spleen, colon, and the capillary endothelium of a variety of tumors. In the prostate, a cytosolic isoform termed PSMA' (produced by alternative splicing) is abundant in normal tissue; the membrane-bound PSMA-1 (prostate-specific membrane antigen, different from prostate-specific antigen PSA!) is found in primary prostate tumors. As physiological functions, GPPII/NAALADase I is required for the uptake of folate in the intestine, and in the brain it modulates

excitatory neurotransmission through the hydrolysis of the neuropeptide *N*-acetylaspartylglutamate (NAAG), thereby releasing glutamate. PSMA is used as a diagnostic and prognostic indicator of prostate cancer and as a possible marker for various neurological disorders such as schizophrenia, Alzheimer's disease, and Huntington's disease. GPPII/NAALDase I inhibitors have analgetic (Yamamoto *et al.*, 2001) and neuroprotective effects (Williams *et al.*, 2001). NAALDase II and III are similar to the former enzyme (Pangalos *et al.*, 1999).

Dipeptidylpeptidase-like protein (DPP6) exists in two alternative splice forms, DPPX-L and DPPX-S, and is a type II transmembrane protein (Wada *et al.*, 1992). Due to the substitution of an aspartate residue for the serine residue in the proposed catalytic triad, it has no dipeptidyl aminopeptidase activity. DPP6 is expressed predominantly in the brain and may be involved in brain function.

Dipeptidyl peptidase 10 (DPP10) is another type II transmembrane protein of 796 amino acids with high (32%) homology to DPP IV (Qi *et al.*, 2003). However, the active serine is replaced by a serine and therefore DPP10 lacks dipeptidyl peptidase activity. The protein is mainly expressed in brain and pancreas.

IV. Conclusions and Perspectives

Most cell-surface (oligo)peptidases have been initially characterized as digestive or clearing enzymes although they cleave only peptides upto 70–80 residues. They are now regarded as regulatory proteases that control the activation or inactivation of circulating hormones and neuropeptides. Consequently, some of them (ACE, NEP, ECE, DPP IV, and in the future probably APP, CPM, and others) are validated drug targets. Inhibitors (or peptidase-resistant analogues) are already widely used as drugs or are under development, mostly for hypertension and diabetes type 2. Future areas appear to be the treatment of pain or inflammatory diseases. Despite the widespread or proposed clinical use of inhibitors, little is known about defective mutations of cell-surface peptidase genes or about the regulation of their expression in disease.

Cell-surface peptidases have also attracted attention in diagnosis, especially for the differentiation of hematopoietic cells and leukemias, although their distribution is more widespread. Endothelial cells are frequent localizations corresponding to their role of inactivation enzymes for hormones. Here, more work is needed to explore their expression in different types of vessels and their probable functional or diagnostic role in angiogenesis.

Research in the past years has made it evident that the integral membrane-bound peptidases belong to gene families that include soluble, often similar peptidases, as well as further integral membrane proteins that are either proteases of different specificity or act as binding proteins. Similar, nonenzymatic functions as binding proteins have also been discovered for some cell-surface peptidases, and there is some evidence that they participate in cell invasion, adherence, and even in signal transduction of other receptor proteins. It appears that these nonenzymatic functions are mostly not related to the catalytic site and other domains of the molecules are involved. Future research is needed to identify further binding targets and especially the physiological relevance of these interactions.

In conclusion, cell-surface peptidases are the key regulators of the inactivation or activation of hormones and neuropeptides. Although most are peptide bond specific, not peptide specific, inhibitors for some of them have become or will become drugs. Also, knowledge concerning their specificity and action is important for the design of peptidase-resistant regulatory peptides. An emerging field of research is their role as binding proteins, especially in cell adherence or invasion.

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