Ecto-enzymes: physiology meets pathology

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Abstract: Ecto-enzymes are catalytic membrane proteins with their active sites outside the cell. They include cholinesterase, which inactivates acetylcholine, and angiotensin-converting enzyme, which converts angiotensin I to biologically active angiotensin II, and numerous other peptidases, transpeptidases, nucleotidases, phosphodiesterases, and phosphatases. Many CD antigens of leukocytes are ecto-enzymes; some CD antigens for which no function is currently known are probably ecto-enzymes. Expression is highly regulated and correlated with differentiation and activation. Some are highly restricted in distribution; others are ubiquitous. Many are shared between leukocytes and non-hematogenous cells. Biological functions appear to depend on the type and location of the cell in which expression occurs, and include recycling of nutrients, local control of response to cytokines and hormones, bone formation, cell mobility, invasion, and metastasis. Many novel regulatory functions of ecto-enzymes remain to be discovered, and may reveal new mechanisms of local extracellular control of cellular function. *J. Leukoc. Biol.* 67: 285–311; 2000.

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INTRODUCTION

The concept of ecto-enzymes is not new. However, it is only recently that this class of membrane protein has become the subject of widespread interest. It is now apparent that there is a large and diverse class of catalytic membrane proteins with their active sites outside the cell. They mediate a variety of biological functions, and can now be seen to represent a new and previously unsuspected level of cellular regulation [1–5].

There are several reasons why the field had a slow beginning [6]. When the idea of ecto-enzymes was first proposed [7], the technology for analysis of the orientation of membrane proteins was inadequate to provide definitive proof that the active site was really outside the cell. Moreover, it was often difficult to conceive of relevant extracellular substrates. There was also some difficulty in accepting the idea that the relevant substrates might include highly abundant intracellular compounds, such as ATP, that perform general metabolic tasks, but that might have radically different and highly specialized functions outside cells [8].

The field is now moving into an exciting phase of rapid development. Ecto-enzymes are being shown to mediate local control of many aspects of cellular function, including response to hormones and cytokines, modulation of the activity of adjacent cells, and control of growth, differentiation, and movement [4].

The topic can be introduced by examples where the biology is well understood. One of the first ecto-enzymes to be identified was cholinesterase, which catalyzes the cleavage of acetylcholine at synapses, thereby terminating cholinergic signaling. Inhibition of cholinesterase results in over-activity of cholinergic synapses, and has been applied in such diverse areas as diagnosis and treatment of myasthenia gravis and chemical warfare against insects and people.

Ecto-enzymes are commonly located on the brush borders of intestinal cells, where they have a general digestive role, but the same enzymes may play highly specific regulatory roles in other cell types. One ecto-enzyme whose function is reasonably well understood is angiotensin-converting enzyme (ACE; dipeptidyl carboxypeptidase; CD143), which converts the inactive prohormone angiotensin I to the biologically active angiotensin II. ACE is present in many locations in the body, notably the vascular endothelium in the lung and brain [9], and is the target of the widely used ACE-inhibitor drugs used to treat hypertension.

Although it is not an ecto-enzyme, 11 β -hydroxysteroid dehydrogenase (11-HSD) exemplifies the principle of how a local cellular enzyme can allow a cell to respond selectively to a hormone in the presence of a vastly higher concentration of another hormone that binds to the same receptor. Cortisol and aldosterone bind to the mineralocorticoid receptor with similar affinity, but cortisol is selectively destroyed by 11-HSD in mineralocorticoid-responsive cells. In other words, selective response to mineralocorticoids is not mediated by an appropriately specific receptor, but rather by the presence of a local enzyme that inactivates a competing molecule [10–12].

ACE and cholinesterase (and to some extent 11-HSD) epitomize some of the more interesting properties of ecto-enzymes. In addition to roles in nutrient uptake, they often act locally to activate or inactivate potent biological mediators. At this time, the biological roles of many ecto-enzymes are less clear. The biggest challenge for the future is to identify the physiologically relevant substrates, and this will be a recurring theme in this review.

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As a general rule, ecto-enzymes are large glycoproteins [13] (see **Fig. 1**). They are commonly type II integral membrane proteins, with a short amino-terminal cytoplasmic domain, a single transmembrane domain, and a large extracellular carboxy-terminal catalytic domain [6]. However, some are type I integral membrane proteins with typical amino-terminal cleavable signal sequences, large extracellular amino-terminal catalytic domains, single transmembrane domains, and short carboxy-terminal cytoplasmic domains. In the case of the ecto-ATPase CD39, there are two transmembrane domains, one at the amino-terminal region and one at the carboxy-terminal region, and the extracellular catalytic loop lies between them (**Fig. 1**). In others, there are multiple transmembrane loops. Alkaline phosphatase and 5' nucleotidase are held in the membrane by a glycosyl phosphatidyl inositol (GPI) anchor [14, 15]. Ecto-

enzymes are often dimeric or multimeric, but are usually made up of a single type of chain.

Many ecto-enzymes also exist in water-soluble forms that are "secreted" from the cell as "exo-enzymes" [16–18]. Release usually appears to occur by hydrolytic cleavage from the membrane, but in some cases closely related genes encode secreted or soluble forms [19, 20]. The possibility also exists that secretion could result from cleavable signal sequences generated by alternative RNA splicing and/or alternative exon usage, as for the membrane and secreted forms of antibodies [21, 22].

Before turning to specific examples of ecto-enzymes, let us consider a few other general points. In principle, it would seem easy and informative to determine the tissue and cellular distribution of ecto-enzymes. These studies have shown that expression is associated with cellular differentiation and/or activation. As might be expected, some ecto-enzymes are expressed in many tissues, whereas others are highly restricted in their distribution [23, 24].

Despite the apparent ease with which the distribution of ecto-enzymes can be studied, there are some subtle difficulties. What was thought to be a single enzyme, or the product of a single gene, has often turned out to be a member of a family of closely related enzymes, the members of which differ in properties such as fine specificity or intracellular targeting [25]. Isoenzymes may be encoded by closely related genes, or may arise by alternative RNA splicing or posttranslational modification. Distinction of individual members of large families of closely related receptors will require reagents with a very high degree of specificity, such as carefully selected monoclonal antibodies. In many cases, such reagents are still lacking.

Even when all these factors have been taken into account, the study of the distribution of individual enzymes has sometimes been disappointing. It has revealed few functional insights concerning their role in physiology. The assumption, implicit but often unstated, is that the distribution will make biological sense. Unfortunately, this has often turned out to be untrue. For example, the ecto-phosphodiesterase/pyrophosphatase PC-1 is present on plasma cells, macrophages, distal convoluted tubule of kidney, liver, epididymis, and chondrocytes, but when the gene is deleted from the germline, the only obvious pathology is in bone and cartilage, and most of the other tissues seem unaffected [26, 27]. Similarly, the intracellular tyrosine kinase *src* is widely expressed, with particularly strong expression in brain, and yet when the gene is deleted from the germline, the major pathology is osteopetrosis [28].

The nomenclature for ecto-enzymes is a mess [20, 25, 29]. There are often multiple names for the same enzyme, reflecting the untidy, chaotic, and nonlinear process of scientific discovery. Order comes about gradually, and only after a certain amount of knowledge has accumulated. In the meantime, names have to be given. Subsequent developments often make them unsatisfactory, but they are not easily changed.

Attempts are being made to simplify and rationalize the nomenclature, but meetings to discuss nomenclature tend to be stormy, and it has often been difficult to reach agreement. Older names persist for good reasons, such as the need to provide a link to previous literature. Abandonment of older names might

Fig. 1. Structure of selected ecto-enzymes. There are many variations on these themes. Many ecto-enzymes are homodimers, although this is not shown. CD26 and CD38 are typical class II membrane proteins, with a membrane/signal sequence near the amino terminus, which is in the cytoplasm. The prototypic structure of CD39 is shown, with transmembrane regions at the amino terminus and the carboxy terminus, which resembles the topography of P2X receptors (see **Fig. 3**). Variants of CD39 are known that have only amino-terminal or only carboxy-terminal transmembrane domains, or even no transmembrane regions at all. The three-dimensional structure of RT6 is known. It contains a conserved "Pacman fold" in the NAD⁺-binding domain, where two abutting β -sheets form the upper and lower "jaws" of the active site cleft. RT6 and CD73 (as well as alkaline phosphatase) are attached to the membrane via a glycosylphosphatidylinositol anchor attached to the carboxy terminus. In some cases, water-soluble forms are also present. Most of these appear to arise by cleavage of the membrane anchor by proteases or phospholipases.

result in searches of the literature failing to identify key papers, and the thread of discovery could be lost. The trivial name that was first assigned to an enzyme is usually easier to remember than the current “random walk through the alphabet.”

The older names are often frankly misleading. Assignment of a name to a family of enzymes does not imply that it has the monopoly on a particular activity. One major group (the CD39 family) is generally known as the “ecto-ATPase” family, but many other enzymes also degrade ATP, including the ecto-pyrophosphatase/phosphodiesterase family [25]. The plasma cell membrane antigen PC-1 is by no means specific for plasma cells. For clarity, I have continued to use some older names for enzymes in this review, but I have cross-referenced them extensively.

Ecto-enzymes may be divided into two main classes: nucleotidases and related enzymes and peptidases (**Table 1**). Let us now turn to specific examples. We will reconsider the basic principles at the end of this review.

ECTO-PHOSPHODIESTERASE/
PYROPHOSPHATASE: THE PC-1 FAMILY

History

It had been known since the early 1950s that the plasma membranes of liver and other tissues contain enzymic activities that hydrolyze nucleotide pyrophosphate and phosphodiester bonds. Evidence gradually accumulated suggesting that these activities might be properties of the same enzyme [30–33].

A second line of work, apparently unrelated, began in the 1960s, with the visionary work of Old, Boyse, and Takahashi, whose first attempts to characterize the lymphocyte surface started long before the advent of monoclonal antibodies. Their strategy was to immunize mice with lymphocytes from different strains and look for alloantisera that recognized subpopulations of lymphocytes.

In 1970 they discovered the plasma cell antigen Pca-1 (later known as PC-1) [34]. Subsequent work showed that the PC-1 antigen had a molecular weight of about 110,000 [35], and that the protein was a disulfide-bonded homodimer [36]. The complete amino acid sequence for mouse PC-1 was determined from cDNA clones in 1987 [37, 38], revealing a type II transmembrane protein, but its function remained unknown. The human PC-1 cDNA sequence is highly conserved [39].

Convergence of the worlds of the enzymologist and the immunologist only occurred in 1991, when Rebbe et al. purified ecto-phosphodiesterase from mouse plasmacytoma cells and discovered its identity with PC-1 [40]. They went on to prove that the nucleotide phosphodiesterase and pyrophosphatase activities were properties of the same polypeptide [41], and postulated that the enzyme might have a role in setting the concentrations of nucleotide sugars in the endoplasmic reticulum and Golgi.

It is ironic that the key to the function of PC-1 had been sitting in the databanks since 1985, when Culp et al. [42] determined the amino acid sequence of the active site of bovine intestinal phosphodiesterase. I had failed to notice the similarity at the time because I had neglected good advice to search all

TABLE 1. Ecto-nucleotidases, ADP-Ribosyl Transferases, and Related Enzymes

Catalytic activity	CD number, aliases, abbreviations	Selected biological activities
Ecto-ADP-ribosyl cyclase; ecto-NADase	CD38	Knockout mice have minor changes in immune response. Enzyme should be classified as a typical NADase. Lymphocyte activation. Cell-cell adhesion?
Ecto-ATPase (EC 3.6.1.5)	CD39, apyrase	Hydrolysis of ATP to ADP and AMP; hydrolysis of ADP to AMP. Conversion of platelet pro-aggregatory ADP into anti-aggregatory adenosine.
Ecto-5' nucleotidase (EC 3.1.3.5)	CD73, lymphocyte vascular adhesion protein 2 (VLAP-2)	Conversion of AMP to adenosine. T cell activation. Cell-cell adhesion?
Phosphodiesterase I (EC 3.1.4.1)/nucleotide pyrophosphatase (EC 3.6.1.9)	PC-1, alkaline phosphodiesterase. Several other family members (see Table 3).	Conversion of ATP to ADP + Pi; conversion of ATP to AMP + PPi; autotaxin can also convert AMP to adenosine, which affects cell motility. Important role for PC-1 in bone formation. Over-expression of PC-1 may cause insulin resistance (controversial).
Ecto-ADP-ribosyl transferase (EC 2.4.2.31)	ART1-7 ART2 = RT6, which has two active copies in the mouse, but one active copy in the rat, and one pseudogene in humans.	T cell activation. Modification and regulation of activity of extracellular domains of membrane proteins including LFA-1 and CD8 and soluble peptides including FGF and tuftsin. Modulation of p56 ^{lck} kinase activity. ART 5 and 7 are secreted.
Alkaline phosphatase (EC 3.1.3.1)	Tissue nonspecific alkaline phosphatase; three other genes are known with different patterns of expression	Can convert PPi to Pi. Deletion of both copies produces severe bone disease (hypophosphatasia)
Acetylcholinesterase (EC 3.1.1.7)		Termination of cholinergic signaling

the databases, and not just Genbank. The vital piece of information was only in the protein database.

In 1991, the strong similarity between PC-1 and the sequence of a peptide from the active site of bovine intestinal phosphodiesterase was also noted by Skinner [43], and it rapidly became apparent that PC-1 and phosphodiesterase I were essentially identical. However, it took several years more research to reveal that the phosphodiesterase from bovine intestine was not “bovine PC-1,” but rather the product of a closely related gene that is expressed in the intestine [25].

The intracellular phosphodiesterases are largely concerned with regulation of cellular signaling rather than general metabolism, and it seems likely that the major roles of the ecto-phosphodiesterases will also be found to be regulatory rather than metabolic.

The E-NPP (PC-1) family of ecto-nucleotide pyrophosphatase/phosphodiesterases

It is now clear that the plasma cell membrane antigen PC-1 was the first member of a multi-gene family of at least three ecto-phosphodiesterase/pyrophosphatases that have been implicated in a wide range of biological activities, including bone formation, cell motility, tumor metastasis, and resistance to insulin in type II diabetes mellitus. At least two additional genes have been identified that contain motifs closely related to the PC-1 active site. Each of these may represent the first members of an extended family.

When PC-1 cDNA was first cloned in 1985 [37], Southern blotting at low stringency suggested a single gene. This view had to be modified in 1994, when a second family member (named autotaxin or PD-1 α) was cloned independently in the human [44] and rat [45], respectively. The following year, a third member was identified in the rat and named gp130^{RB13-6}

[46]. A splice variant of this gene encoding a form of the enzyme expressed in rat liver (B10) was identified in 1997, and the corresponding human cDNA (named PD-1 β or PDNP3) was independently cloned in the same year [47]. The current situation is summarized in **Table 2**.

Nomenclature of the E-NPP (PC-1) family

Nomenclature for the ecto-pyrophosphatase/phosphodiesterases was considered at the Second International Workshop on Ecto-ATPases and Related Ectonucleotidases held in Belgium in June 1999 [29]. Designation as 5'-nucleotide phosphodiesterases or phosphodiesterases-1 appears no longer advisable because the terms were initially coined to differentiate these enzymes from 3'-exo(deoxy)ribonucleases (EC 3.1.16). They should not be classified as exonucleases because, unlike canonical exonucleases, they act much better on oligonucleotides than on polynucleotides. The name NPP (i.e., nucleotide phosphodiesterase/pyrophosphatase) was preferred because it indicates the specificity for nucleotides and not for lipid phosphodiesterases.

It was agreed that the enzyme family should be designated E-NPP (i.e., ecto-nucleotide phosphodiesterase/pyrophosphatase), and the enzymes numbered E-NPP1, 2, 3, etc. in order of date of publication. Numbers should not be definitively assigned until enzymic activity is demonstrated. Splice variants are to be given the Greek suffix α , β , etc. The first enzyme of the family to be identified (PC-1) would be designated E-NPP1. The second (autotaxin or PD-1 α) would be designated E-NPP2, and the third (gp130^{RB13-6} or PD-1 β or B10) would be designated E-NPP3.

Nomenclature for the relevant genes is somewhat different. Human genes have a mandatory four-letter code and are italicized in upper case. The corresponding genes in other

TABLE 2. The Ecto-phosphodiesterase/Pyrophosphatase Family

Protein (aliases)	Preferred name	Gene	Distribution/and function
PC-1 (phosphodiesterase I; PDNP1; NPP γ)	E-NPP1 (nucleotide pyrophosphatase/phosphodiesterase 1)	<i>Npps</i> <i>PDNP1</i>	Mouse: plasma cells, macrophages, brain capillaries, hepatocytes (basolateral), epididymis, salivary duct epithelium, distal convoluted tubule of kidney. Human: requires further study. Expressed in pancreas, uterus, heart, placenta, prostate, liver, testis; low levels in skeletal muscle, thymus, prostate.
Autotaxin (PD-1 α ; PDNP2, NPP α)	E-NPP2	<i>PDNP2</i>	Human: brain, small intestine, lung, kidney, placenta, ovary. Rat: brain; absent from small intestine.
B10 (gp130 ^{RB13-6} ; PD-1 β ; PDNP3; bovine intestinal phosphodiesterase; NPP β)	E-NPP3	<i>PDNP3</i>	Human: prostate, uterus, absent from small intestine, liver, or pancreas. Rat: small intestine, hepatocytes (apical), exocrine pancreas.
KIAA 0879 (Genbank accession no. AB020686)	E-NPP4?	<i>PDNP4?</i>	Human brain. Phosphodiesterase activity remains to be demonstrated.
GPI7 (YJL062w)		Found in yeast. May exist in human (NCBI protein identification no. 2984587).	Phosphodiesterase activity remains to be demonstrated; adds side chain to GPI anchor.

species are given in lower case. At the present time, the gene for mouse PC-1 is named *npps*, but this name will have to be changed because we now know that there are at least three genes. The human gene for PC-1 has been referred to as *NPPS* [48], although it is better known in the literature as *PDNPI* [47].

Enzymic activities

Ecto-phosphodiesterases are distinct from the intracellular phosphodiesterases. They have broad specificity, and are capable of hydrolyzing phosphodiester bonds of nucleotides and nucleic acids, and pyrophosphate bonds of nucleotides and nucleotide sugars [20]. They can hydrolyze nucleoside 5'-triphosphates such as ATP to AMP and PP_i, nucleoside 5'-diphosphates such as ADP to AMP and P_i, and NAD⁺ to AMP and nicotinamide mononucleotide. They can also hydrolyze diadenosine polyphosphates and 3',5'-cAMP to AMP. Although they are often regarded as being involved with hydrolysis of adenine nucleotides, they can also hydrolyze GTP, TTP, CTP, and UTP with similar efficiency. They may be regarded as extracellular ATPases, although for purely historical reasons this term is usually confined to members of the CD39 family.

Autotaxin seems to have the broadest activity. In addition to cleavage of ATP to AMP with release of pyrophosphate (PP_i), it can cleave ATP to ADP with release of orthophosphate (P_i), and can cleave ADP to AMP with release of P_i, and also cleave PP_i to 2 P_i. It appears to be the only family member that can cleave AMP to adenosine and P_i, in a similar manner to 5' nucleotidase (CD73).

PC-1 is also capable of autophosphorylation, and has been claimed to be an ecto-protein kinase that is capable of phosphorylating other proteins [49–54]. These aspects of PC-1 will be discussed in a subsequent section on ecto-protein kinases.

Structure of the PC-1 family of ecto-phosphodiesterases

The structure of the PC-1 family members is shown in composite form in **Figure 2**. There is a short amino-terminal cytoplasmic tail, which may be involved in intracellular targeting (see below), a single transmembrane region, and a large carboxy-terminal extracellular catalytic domain, which contains a completely conserved threonine residue that is transiently adenylated and/or phosphorylated during the catalytic cycle [18, 42, 53, 54]. The active site threonine (underlined) is contained in a motif PTXTX₈TGX₂P that is common to mammalian, viral, plant, and bacterial phosphodiesterases [55].

The catalytic domain is attached to the membrane by a “stalk” consisting of two cysteine-rich motifs that are closely related to a putative growth factor called “somatomedin B.” (The notion that somatomedin B is a growth factor has not withstood the test of time, but the name persists). Close to the carboxy terminus there is an EF hand motif [56] that binds divalent cations and stabilizes the protein [57], and is required for enzymic activity [58].

There is an RGD (Arg-Gly-Asp) motif in the first somatomedin B repeat in E-NPP2 (autotaxin; PD-1α), which is conserved in rat and human, and an RGD motif in the second somatome-

Fig. 2. The PC-1 family and related proteins. (A) Composite picture of the three most well-characterized members of the PC-1 family. The position and number of carbohydrate moieties varies somewhat between family members. T represents the active site threonine. The RGD sequence is present in the first somatomedin B repeat (SMB1) in E-NPP2 (PDNP2; autotaxin; PD-1α) of rat and human, but is not present in the second somatomedin repeat. In contrast, the RGD sequence is present in the second somatomedin repeat (SMB2) of E-NPP3 (gp130^{RB3.6}; B10; PD-1β; ?PDNP3) of both rat and human, but not in the first repeat. Neither human nor mouse PC-1 has an RGD sequence. The site of proteolytic cleavage of human autotaxin (E-NPP2) is shown by the arrow. (B) The sequence of an expressed sequence tag (EST) from human brain (KIAA 0879; Genbank accession number AB020686) suggests a type I membrane protein with a high degree of identity with the PC-1 family in the region of the active site. The orientation is the opposite of the PC-1 family. This protein is tentatively designated E-NPP4, although at the present time enzymic activity has not yet been demonstrated. (C) Yeast membrane protein *GPI7*, which is required for addition of a side chain to GPI anchors and affects the transport of GPI-linked proteins. It has a conserved N-region motif (PTXTX₈TGX₂P) that is present in the active sites of bacterial, viral, plant, and mammalian phosphodiesterases, although phosphodiesterase activity has not yet been demonstrated. It is possible that it is actually a transferase. Hydrophobicity plots suggest that the amino-terminal hydrophilic region containing the phosphodiesterase active site motif is extracellular, but the precise arrangement of the transmembrane domains is not yet clear.

din B repeat in NPP3 (B10; PD-1 β), which is also conserved between rat and human. E-NPP1 (PC-1) does not have an RGD sequence at either site. RGD sequences in extracellular matrix proteins have been found to mediate adhesion with cell-surface integrin molecules [59]. The fact that these two RGD sequences are conserved between species and in analogous positions in the two somatomedin B domains may be significant.

It is tempting to speculate that these conserved cell-surface RGD motifs are functionally important in binding to other proteins. Some evidence for this has recently been obtained. The transferrin receptor has a highly conserved RGD sequence in its transferrin-binding domain, and the RGD motif is essential for binding to transferrin [60]. The RGD motif is also present in the homologous position in a recently described second transferrin receptor gene [61].

Polymorphism of the PC-1 gene

PC-1 has two alleles in the mouse. PC-1^a is found in BALB/c, C3H, CBA, A/J, AKR, NZB, and SJL mice, and PC-1^b in C57BL and DBA/2 mice. Strain 129 was originally classified as PC-1^b [34], but this "strain" has many sublines that have major genetic differences [62, 63], and 129/Sv appears to be PC-1^a [A. Sali and J. W. Goding, unpublished data]. The structural basis for these allelic forms is not yet known [34], but it is clear that PC-1^b encodes an active protein and is not a "null" allele [64]. Most available monoclonal antibodies to mouse PC-1 recognize only the PC-1^a allele [24, 25, 65, 66]. One monoclonal antibody (4H4) raised against human PC-1 also recognizes both alleles of mouse PC-1, but only recognizes the unfolded protein [57]. Immunization of PC-1 knockout mice with the BALB/c plasmacytoma line MOPC-21 has allowed the development of a new monoclonal antibody (2D7-3) that recognizes both alleles of mouse PC-1 as well as the human protein [A. Sali and J. W. Goding, unpublished data].

Several genetic variations have recently been described in the human PC-1 gene. These are of particular interest because of their association with skeletal abnormalities [48] and insulin resistance in type II diabetes [67] (see below).

Tissue distribution of the PC-1 family

An extensive survey of the tissue distribution of PC-1 in the mouse showed that in addition to expression on plasma cells, it is also present on chondrocytes, hepatocytes, the distal convoluted tubule of the kidney, epididymis, salivary ducts, and capillaries in the brain [24]. No expression of PC-1 was found in the gut in mice. These results are somewhat different to those of Morley et al. [23], who used a cross-reacting rabbit polyclonal antibody directed against affinity-purified bovine intestinal phosphodiesterase to study the distribution of the enzyme in humans. Resolution of this difference came with the discovery of two additional family members, with differing tissue distribution between families and also between species [25].

Paradoxically, E-NPP2 (autotaxin or PD-1 α) is strongly expressed in the small intestine in humans [68] but not in rats [45], whereas the reverse is the case for E-NPP3 (B10; PD-1 β) [47, 69]. These results suggest that these enzymes are capable of substituting for each other in the intestine. It is curious that E-NPP3 is strongly expressed in the liver in the rat, whereas

expression of both E-NPP3 and E-NPP2 is weak or absent in this organ in humans.

Biological properties of PC-1 (E-NPP1; PDNP1; NPP γ)

The function of PC-1 in plasma cells remains to be determined. PC-1 knockout mice seem to have normal gamma globulin levels and are capable of responding to antigen by production of specific antibody [J. W. Goding and A. Sali, unpublished observations]. However, there is overwhelming evidence that PC-1 plays a fundamental role in bone and cartilage, and it may also be involved in the pathogenesis of insulin resistance in type II diabetes.

PC-1 and bone development

The finding that PC-1 is strongly expressed in cartilage was made by Harahap et al. [24]. This observation is of interest because the hydrolysis of extracellular ATP by PC-1 generates pyrophosphate, which has profound effects on calcification of bone. Excessive pyrophosphate may play a role in the pathogenesis of chondrocalcinosis, where articular cartilage becomes calcified [70]. Recent work suggests a complex interplay between the ratio of inorganic pyrophosphate (PPi) and orthophosphate (Pi) in the calcification of bone [71]. PC-1 seems to play a pivotal role in these processes.

The role of PC-1 in bone formation has recently been confirmed by the identification of a point mutation in PC-1 in *ttw/ttw* (tiptoe walking) mice [26]. These mice have extensive calcification of peri-articular tissues and ligaments, leading to joint fusion. An essentially identical picture is seen in mice in which the PC-1 gene has been disrupted by homologous recombination [27]. *Ttw* mice have been used as a model for the human disease known as ossification of the posterior longitudinal ligament of the spine (OPLL), but there are significant differences between the mouse model and the human disease.

The question of whether the gene for PC-1 (referred to as *NPPS*) is involved in the pathogenesis of human OPLL is not yet resolved. At least 10 nucleotide variations have been found in the gene, and five of these result in changes in amino acids. Two were found specifically in OPLL patients [48, 67]. In addition, a single base change in intron 20 was more prevalent in OPLL than in the general population [48]. These results suggest an important role of PC-1 in human OPLL, but the exact mechanism is unknown at this time.

The mechanisms by which the abnormal calcification occurs in mice lacking PC-1 are not completely clear. The great majority of nucleotide pyrophosphohydrolase activity in human osteoblasts and articular chondrocytes can be accounted for by PC-1 [72, 73]. Disruption of the PC-1 gene would be expected to alter the ratio of PPi to Pi in these tissues. Because PPi can inhibit the formation and propagation of hydroxyapatite crystals *in vitro* [74], removal of this inhibition by inactivation of PC-1 might promote excessive calcification.

PC-1 might also exert effects on bone formation that are distinct from effects on phosphate metabolism. Osteoblasts and chondrocytes express P2 purinergic receptors that are activated by extracellular ATP, ADP, or related molecules [75]. These P2

ligands might accumulate to abnormally high levels when the PC-1 gene is disrupted.

PC-1 and insulin resistance

An intriguing but controversial role has been postulated for PC-1 in the pathogenesis of type II diabetes mellitus [76], which is associated with resistance to the action of insulin by unknown mechanisms [77, 78]. Fibroblasts from patients with severe insulin resistance were found to have elevated expression of PC-1 [79], and transfection of cells with PC-1 caused them to acquire resistance to the action of insulin. Surprisingly, insulin resistance did not seem to require the enzymic activity of PC-1, nor did it appear to involve generation of adenosine [80]. The mechanism of these effects is not yet understood, but a physical association between PC-1 and the insulin receptor has been suggested.

Substitution of glutamine for lysine at position 121 of human PC-1 (K121Q) has been found to be associated with insulin resistance in a population of Caucasians in Sicily [67]. This interesting and provocative finding will need to be explored in other populations, but seems to add strength to the case for a causal role of PC-1 in diabetes.

However, the role of PC-1, if any, in insulin resistance has been highly controversial [81–83]. It has been suggested that the inhibition of phosphorylation of the insulin receptor by PC-1 is an *in vitro* artifact due to destruction of ATP [81], but this mechanism obviously cannot apply in the case where the enzymic activity of PC-1 has been abolished by mutagenesis of the active site threonine [80].

PC-1 knockout mice seem to have normal blood glucose levels, as do transgenic mice in which the PC-1 gene is overexpressed. At this time, no definitive conclusions can be drawn concerning any role for PC-1 in type II diabetes. Resolution of these issues will require further research.

PC-1 and hydrolysis of diadenosine polyphosphates

PC-1 is also capable of hydrolysis of diadenosine polyphosphates, which are present in the storage granules of a number of secretory cell types. Diadenosine polyphosphates have been proposed to act as extracellular signaling molecules through their action on P2 receptors, which can mediate vasoconstriction, vasodilatation, platelet aggregation, synaptic transmission, apoptosis, and cell proliferation. The membrane enzyme that hydrolyzes diadenosine polyphosphates in the bovine adrenal medulla appears to be a member of the PC-1 family, possibly PC-1 itself [84]. Harahap et al. found no evidence for expression of PC-1 in the adrenal in mice [24], but this point should be re-examined to see whether it reflects species differences or whether a different family member is present in the mouse.

Biological properties of autotaxin (NPP2; PDNP2; PD-1 α)

Autotaxin is the name given to a biological activity found in the supernatants of human A2058 melanoma cells, which promotes cellular motility. The activity was classified as a cytokine. The motile response of cells was abolished by pretreatment with pertussis toxin, suggesting that the cytokine acted via a

G-protein-coupled receptor. Cloning of autotaxin cDNA was achieved in 1994 and, surprisingly, revealed high homology with phosphodiesterases and PC-1 [44]. In the same year, cDNA encoding an ecto-phosphodiesterase was cloned from rat brain and named PD-1 α [45]. It soon became apparent that these cDNAs were splice variants of the same gene. Several other splice variants have now been cloned from rat brain [85]. Autotaxin cloned from human teratocarcinoma cells was found to be almost identical to autotaxin cDNA from melanoma cells [68], but lacked a 52-amino-acid insertion that was present in the latter. PD-1 α also lacks the 52-amino-acid insertion that was present in the human melanoma cells, but contains a second insertion of 25 amino acids that is lacking in both the human autotaxins.

Autotaxin is highly expressed in brain in both humans and rats, and in small intestine in humans but not in rats. In humans, it is also expressed in lung, kidney, placenta, and ovary.

As defined biologically, autotaxin is a water-soluble protein, although the cDNA predicts a type II membrane protein. Autotaxin appears to be generated by proteolytic cleavage from the membrane between Ser⁴⁸ and Asp⁴⁹, which is adjacent to the extracellular surface of the membrane (see Fig. 2). Soluble forms of PC-1 have also been described [17]. It was postulated that the cleavage site of PC-1 is between Arg¹⁶⁹ and Asp¹⁷⁰ (between the second somatomedin B domain and the active site domain), but this has not been tested by protein sequencing and may be incorrect.

As mentioned earlier, the enzymic activity of autotaxin is broader than that of other members of the PC-1 family. It is able to hydrolyze ATP, ADP, AMP, and even PPi, and unlike PC-1 and PD-1 β , can generate adenosine. This last property is particularly important because the biological activity of autotaxin requires its phosphodiesterase activity [86]. There is strong evidence that its motility-inducing activity is mediated via the binding of adenosine to P1 adenosine receptors [87] because motility is stimulated by adenosine agonists and is blocked by adenosine antagonists. Moreover, responsiveness can be transferred into Chinese hamster ovary cells by transfection with cDNA encoding the adenosine A1 receptor. These interesting results raise the possibility of novel therapeutic agents against tumor metastasis.

Biological properties of B10 (NPP3; PDNP3; PD-1 β ; gp130^{RB13-6})

The third member of the PC-1 family was identified independently by three groups. A protein that was present on the bile canalicular surface of rat liver was detected by a monoclonal antibody and named B10 [88, 89]. The corresponding cDNA was cloned from fetal rat or human brain and named gp130^{RB13-6} [46] or PD-1 β [90]. Unlike PC-1, gp130^{RB13-6} does not appear to be a disulfide-bonded dimer [46], although noncovalent dimerization seems likely. Soluble forms of B10 (PD-1 β) have also been shown to exist [91].

The tissue distribution of NPP3 differs considerably between species. In humans, it is present in the prostate and uterus, but is absent from small intestine and liver. In the rat, it is strongly

expressed on the bile canalicular surface of liver and on the brush border of enterocytes of the small intestine [69].

So far, no clear biological function has been found for this member of the PC-1 family, although it might play a role in hydrolysis of nucleotides in the gut lumen, converting them into a form that can be absorbed. It is also expressed in bone, and in view of the dramatic effects of deletion of PC-1, it will be of interest to see whether deletion of B10 (PD-1 β) causes any changes in calcification.

Intracellular targeting signals for ecto-phosphodiesterases: conservation and divergence of the amino acid sequences of the transmembrane and cytoplasmic tails

The distribution of ecto-phosphodiesterases in rat liver is striking. PC-1 is present exclusively at the basolateral membrane, whereas B10 is exclusively at the apical (bile canalicular) membrane [69, 89]. The biological relevance of this distribution is not yet clear, but it raises an interesting question. How and why do two closely related proteins with apparently identical enzymic activities become localized to opposite poles of the same cell?

Comparison of the sequences of B10 and PC-1 is informative. Their extracellular catalytic domains are very similar [25] and the transmembrane and cytoplasmic sequences are very highly conserved between species within each group (**Table 3**), but the transmembrane and cytoplasmic domains of B10 and PC-1 show no detectable homology, and also differ greatly from those of autotaxin (Table 3).

Comparison of the cytoplasmic tails of human and mouse PC-1 is also striking (Table 3). The lengths of the tails are different (58 and 76 amino acids, respectively), and it is

necessary to introduce three large gaps to maximize the homology. This discrepancy led to some confusion concerning the identity of the initiator methionine, but it is now definitively established that the first methionine in each sequence is the initiator [92]. In spite of the differences in length of the cytoplasmic tails, there are “islands” of very strong conservation, presumably reflecting functional constraints. One such constraint is likely to be the presence of targeting signals that direct the protein to the appropriate cell surface. The presence of these conserved islands suggests that there may be a biological penalty for genetic drift in this part of the protein, and that basolateral expression of PC-1 serves a useful role.

The logical place to seek the signals for basolateral location of PC-1 in polarized epithelia would be in the regions that are conserved between species. A strong candidate for signals for basolateral location of PC-1 is the Leu-Leu sequence in the cytoplasmic tail, because this motif has been found to mediate basolateral location in other proteins [93, 94]. It is conserved between mouse and human PC-1 (Table 3).

The signals for apical location of membrane proteins in polarized epithelia are less clear. There is evidence that N-linked glycans can provide such a signal [95], although it can probably be overridden by stronger signals contained within the cytoplasmic tail.

It is also conceivable that the transmembrane regions could contain targeting information. The transmembrane sequences of PC-1 and B10 differ greatly from each other, but are highly conserved between species, suggesting that the transmembrane region requires more than just conservation of hydrophobicity (Table 3). Possible constraints could include targeting signals within the transmembrane region and/or dimerization of sub-

TABLE 3. Cytoplasmic Tails and Transmembrane Regions of the PC-1 Family of Ecto-phosphodiesterases

Enzyme	Species	Amino acid sequence
E-NPP1 (PDNP1; PC-1) CYTO TAIL	Mouse	MERDG-----DQAGHGPRHGSAGNGRE----- ***** * *+ * * *****+
	Human	MERDGCAGGSGRGEGGRAPREGPAGNGRDRGRSHA
	Mouse	LESP----AAASLLAPMDLGEPELEKAERARPAKDPNTYK * * *****+***** * * *****
E-NPP2 (PDNP2; autotaxin) cyto tail	Human	AEAPGDPQAAASLLAPMDVGEEPLEKAARARTAKDPNTYK
	Rat	MARQGCLGSFQ *** + * *
E-NPP3 (PDNP3; B10) cyto tail	Human	MARRSSFQSCQ
	Rat	MDSRLALATEEPIKKDSLKRYK *+* * ***** *+*+ *+*+*
E-NPP1 (PDNP1; PC-1) trans-membrane	Human	MESTLTLATEQPVKKNTLKKYK
	Mouse	KVLSVLVSVCVLTITILGCIFGLK *****
E-NPP2 (PDNP2; autotaxin) trans-membrane	Human	KVLSVLVSVCVLTITILGCIFGLK
	Rat	QVISLFTFAISVNICLGFTASR *+*****+ * *****
E-NPP3 (PDNP3; B10) trans-membrane	Human	QIISLFTFAVGVSICLGFTAH
	Rat	KILCAVLLALLVIVSLGLGLGLRL ** * *****+***** *
	Human	KIACIVLLALLVIMSLGLGLGLRL

* Identical amino acids; † conservative substitutions. For each family member, the cytoplasmic and transmembrane domains are highly conserved between species, indicating functional constraints on genetic drift. In contrast, the sequences of the cytoplasmic tails and transmembrane regions differ greatly between family members, most probably reflecting differences in signals that direct intracellular location to apical or basolateral membranes. The sequence of the cytoplasmic tail of mouse PC-1 has been corrected for three errors in the previously published sequence. Optimal alignment of the cytoplasmic tails of mouse and human PC-1 require several gaps, revealing “islands” of conserved sequence. It is likely that these conserved islands contain important signals for intracellular trafficking.

units [21, 22]. Site-directed mutagenesis and the construction of chimeric proteins will allow many of these ideas to be tested.

More distantly related members of the PC-1 family

Recently, a strong candidate for a more distantly related member of the PC-1 family has been discovered. This protein was identified in a large group of cDNAs encoding high-molecular-weight proteins expressed in human brain [96]. The cDNA KIAA0879 (Genbank accession no. AB020686) is predicted to encode a type I membrane protein with a short carboxy-terminal cytoplasmic tail, a single transmembrane region, and a large amino-terminal extracellular domain with up to 10 N-linked glycosylation sites (Fig. 2). It contains the sequence TKTFPNHYSIVTGLY, which is 100% conserved between the active sites of mouse and human PC-1, including the active site threonine (underlined). It seems likely that this protein will be found to have ecto-phosphodiesterase activity. It may represent the first member of a new branch of the ecto-phosphodiesterase family.

Finally, an even more distant relative (*GPI7*) has been identified in yeast [55]. This protein was identified as being required for addition of a side chain to GPI anchors, and it is predicted to be an 830-amino acid transmembrane protein with 5 glycosylation sites and 9–11 transmembrane domains (Fig. 2). Its hydrophilic amino-terminal domain is predicted to be extracellular and contains the motif PTXTX₈TGX₂P, which is common to phosphodiesterases in bacteria, plants, and humans. Homologs have been identified in *C. elegans* and in human (NCBI protein identification no. 2984587), but its precise function is currently unknown, nor is it yet known whether it has phosphodiesterase activity. The significance of the multiple transmembrane regions is unknown.

ECTO-ATPases/APYRASES (CD39 FAMILY)

History

Many intracellular processes use ATP as their source of energy, and because they consume ATP, the relevant enzymes are known as ATPases. When it became apparent that there was a major class of ATPases with their active sites outside the cell, they were designated ecto- or E-type ATPases [reviewed in refs. 20, 29, 97]. Members of this family are also known as Ecto-(Ca²⁺, Mg²⁺)-apyrases.

CD39 was originally identified on the surface of Epstein-Barr virus-transformed B cells, and was subsequently shown to be present on activated B and natural killer (NK) cells and subsets of activated T cells, but not on resting lymphoid cells [98, 99]. In 1996, Wang and Guidotti discovered that CD39 has sequence homology with a potato apyrase, and that CD39 has apyrase activity [100]. This work led to the identification of a family of ecto-ATPases that are related in sequence, but vary in their membrane topology and tissue distribution [20, 97]. The first member of this group, CD39, was found to hydrolyze either ATP or ADP, and was called “ecto-ATP diphosphohydrolase.” Further work has shown that some members of this family have a strong preference for ATP, whereas others also efficiently

hydrolyze ADP. These activities found in soluble enzymes have been generally known as apyrases, but the convergence of ecto-ATPases and apyrases has now rendered these terms more or less interchangeable.

Nomenclature of the E-NTPDase (CD39) family

As is the case for the ecto-phosphodiesterases, nomenclature of the CD39 family has been confusing and misleading. All members of the E-NTPDase family can hydrolyze a variety of nucleoside 5'-triphosphates and nucleoside 5'-diphosphates, and not just ATP and ADP, although preferences can vary considerably between individual enzymes. There is no particular reason why the CD39 family should be called ecto-ATPases while the PC-1 family is not, but the names have stuck.

Nomenclature was considered by a committee at the recent Second International Workshop on Ecto-ATPases and Related Ectonucleotidases [29]. It was agreed that the CD39 family should be referred to as E-NTPDases (Ecto Nucleoside TriPhosphate Diphosphohydrolase; EC 3.6.1.5 apyrases). The term “apyrase” might still be used (EC 3.6.1.5), although it has some limitations.

Members of this family should be named E-NTPDase1, 2, 3, etc. in order of date of publication, but numbers are not definitively assigned until enzymic activity is demonstrated. The same name is to be given to orthologs in different species. Splice variants are to be given the Greek suffix α , β , etc. Nomenclature for the relevant genes is to be given in italicized upper case for human genes (e.g., *NTPD1*), and in lower case for other species.

Structure of the E-NTPDase family

The prototypic structure for the E-NTPDase family consists of an amino-terminal cytoplasmic domain, a transmembrane domain, an extracellular glycosylated catalytic domain, a second transmembrane domain, and a carboxy-terminal cytoplasmic domain [101] (Fig. 1). This unusual topography resembles that of the P2X family of purinoceptors (see Fig. 3). There are numerous variations on this theme, including a single carboxy-terminal transmembrane domain. Although most are present on the cell surface, there are also forms that are present facing the lumen of the Golgi (which is topologically extracellular), as well as soluble forms with no transmembrane domains. Their molecular masses are typically about 70–80 kDa including carbohydrate, and they may exist in higher multimeric forms. Some may be released from the cell surface in enzymatically active form by proteolytic cleavage [20].

Biological properties of the E-NTPDase family

The biological activities of E-NTPDases are still not completely clear, but they are often associated with response to tissue insult or injury. CD39 is expressed on vascular endothelium, and there is a growing body of evidence that it has a role in regulation of blood coagulation. ADP is one of the most potent known agonists for platelet aggregation, and presumably acts via P2 purinoceptors. The physiological protective response to activation or injury of endothelial cells is prothrombotic and accompanied by loss of CD39 expression. Because CD39 destroys extracellular ATP and ADP, ADP concentrations

Fig. 3. Modulation of effects of extracellular nucleotides by ecto-enzymes. The functional specificity of a given receptor depends on activation or inactivation by adjacent enzymes, which could both initiate and terminate signaling. Note the similarity of the transmembrane topography of P2X receptors with that of CD39 ecto-ATPase (see Fig. 1).

would therefore be expected to rise, favoring clotting [102]. It has therefore been suggested that externally administered apyrase might be a useful antithrombotic agent [103].

The recent production of mice in which the CD39 gene is disrupted by homologous recombination has revealed a complex phenotype, with prolonged bleeding times but minimally disturbed coagulation parameters [104]. Platelets from mutant mice showed reduced interaction with injured vasculature and decreased ability to aggregate in response to standard agonists *in vitro*. This dysfunction was reversible and associated with P2Y1 receptor desensitization, presumably in response to chronic stimulation by abnormally high concentrations of ADP.

CD39 is strongly expressed together with CD73 (ecto-5'-nucleotidase) in the inflammatory region surrounding ischemia in the brain. This may represent a co-coordinated response to the release of intracellular ATP caused by injury and might be beneficial in several ways, including the generation of adenosine as a neuroprotective agent, the salvage of purines, and by counteracting the cytotoxic, Ca^{2+} -mediated effects of extracellular ATP on neurons or glial cells [20].

ECTO-NAD GLYCOHYDROLASE/ADP RIBOSYLCYCLASE (CD38)

Structure and distribution of CD38

CD38 is a type II membrane protein that has been used extensively to identify and characterize lymphocyte subsets. It is expressed on a wide range of hematopoietic and nonhematopoietic cells, including thymocytes, B cells, long-term reconstituting stem cells in the mouse, and at low levels on spleen colony-forming cells (CFU-S).

Expression patterns of CD38 differ significantly between mouse and human, particularly in the B cell compartment. In the mouse, CD38 is expressed at low levels in early B cell development, rising to very high levels on mature naive B cells, but its expression is low in germinal centers, plasma cells, and

B cells that have switched antibody isotype. In the human, however, expression of CD38 decreases during B cell development, but is increased in germinal centers and plasma cells. There are therefore difficulties in comparing results between the two species [105].

Biological effects of CD38

CD38 became of major interest with the discovery that certain monoclonal antibodies to it resulted in B cell activation and proliferation, suggesting a role in signal transduction [105]. The effect was highly synergistic with lipopolysaccharide and interleukin-4 (IL-4). CD38 has only a short cytoplasmic tail (23 amino acids) and no obvious signaling motifs, and the mechanism of these effects has been difficult to determine. However, there is ample precedent for ecto-enzymes having signal transduction capacity, because this has been demonstrated for CD26, CD73, and RT-6 (see below).

The situation took an interesting turn with the discovery that CD38 was closely related to ADP ribosyl cyclase (ARC), which converts $\beta\text{-NAD}^+$ into ADP-ribose and cyclic ADP-ribose (cADPR), molecules involved in intracellular signaling in other systems [106]. However, in retrospect the discovery of ARC activity was probably misleading. The active site of the enzyme is outside the cell, whereas the only known location of these products is intracellular. (The precedent of extracellular ATP meant that this objection did not have to be taken very seriously.) Production of cADPR does not seem to be necessary for signaling. More recent work has shown that CD38 should be regarded as a "classical" NAD^+ glycohydrolase [107], and may indeed be the only NAD^+ glycohydrolase in mammals, but it may have little or nothing to do with cyclic ADP-ribose.

The molecular basis of the signaling properties of CD38 is unclear [105]. There seems to be a pathway of extracellular cross talk between CD38 and the B cell receptor, and signaling

via CD38 seems to require the presence of the B cell receptor for antigen as well as the receptor-associated Ig α or Ig β chains.

CD38 has also been implicated in cell-cell adhesion, and appears to bind to CD31, an immunoglobulin superfamily member [108]. A water-soluble form of CD38 has been described [109].

A more prosaic but perhaps more firmly based function of CD38 is suggested by the fact that its expression is co-coordinately up-regulated together with PC-1 and an enzyme that converts AMP into adenosine, but which may be distinct from CD73 [110]. One possibility is that the latter enzyme is autotaxin (see above). Up-regulation of these three enzymes was seen after treatment of human lymphocytes with phorbol esters or other activating agents. The co-coordinated regulation of these enzymes may be significant in recycling extracellular nucleotides because cell death by apoptosis is a very prominent feature of many stages of lymphocyte development in the thymus, in germinal centers, and after antigen activation. A similar role has been proposed for CD73 in the thymus [111].

Knockout mice in which the CD38 gene is disrupted show remarkably few changes. Most immune responses are normal, although there were lower IgM, IgG1, and IgE antibody responses when low doses of antigen were used. No apparent abnormalities in non-lymphoid organs were detected in the knockout mice [112].

Much work remains to be done before we can confidently assign biological roles to CD38 [105, 113, 114].

ECTO-ADP-RIBOSYLTRANSFERASE

History

ADP-ribosylation is a covalent modification of proteins mediated by enzymes that transfer the ADP-ribose moiety from NAD⁺ to an acceptor with release of nicotinamide. The acceptor is usually a protein, and the most commonly modified amino acid is arginine, although histidine and cysteine may also be modified. The reaction was discovered about 30 years ago as the pathogenic mechanism of action of diphtheria toxin, which was found to modify elongation factor 2 at a histidine residue, resulting in the cessation of protein synthesis. Several other bacterial toxins are also ADP-ribosyltransferases. Cholera and pertussis toxins exert their effects by ADP-ribosylating the α subunit of the heterotrimeric G proteins at arginine and cysteine, respectively. Most bacterial ADP-ribosyl transferases are encoded by mobile genetic elements such as phages, plasmids, and pathogenicity islands, and horizontal transfer between species seems likely [115]. The NAD⁺-binding domain is made up of two abutting β -sheets that form the “jaws” of the active site cleft, and this motif has been termed the “Pacman fold” (Fig. 1).

It has been hypothesized that the bacterial toxins mimic endogenous mammalian regulatory enzymes, and there is considerable experimental evidence in support of this. The first family member to be cloned from mammals was the highly abundant nuclear enzyme poly-ADP-ribose polymerase (PARP), which is a substrate for cleavage by caspases during apoptosis. PARP may play a role in DNA excision repair [116, 117]. When

the crystal structure of PARP was elucidated in the 1960s, it became apparent that it was very similar to that of the corresponding bacterial ADP-ribosyltransferase toxins. Subsequently, a series of mammalian ADP-ribosyltransferases (ART) were characterized and cloned, and a new mammalian enzyme family emerged [115, 118, 119].

The RT6 T cell antigen

It was surprising to discover that one of the members of the ART family was the rat T cell alloantigen RT6 [120–122], which is a GPI-anchored membrane glycoprotein [115, 118, 119]. There are now known to be at least six functional RT6-related enzymes in the mouse and four in humans. RT6 corresponds to ADP-ribosyltransferase 2 (ART2). Structural predictions suggest a folding pattern very similar to that of the bacterial toxins [120].

The most complete information concerning expression of RT6 is in the rat, where monoclonal antibodies are available. It is expressed on most mature T cells and some NK cells, but not on thymocytes or recent thymic emigrants. It does not seem to be expressed outside the immune system.

Substrates for RT6

Substrates of the RT6 family have been identified by incubating RT6-expressing cells with radioactive NAD⁺. They include the integrin LFA-1 (α L/ β 2) and CD8, the co-receptor for MHC class I molecules that is present on the surface of cytotoxic T cells and their precursors. LFA-1 strengthens the binding of cytotoxic T cells to their targets, and it has been postulated that ADP-ribosylation is responsible for reduced target cell binding and altered migration behavior of NAD⁺-treated T cells [115, 123].

Another possible target for ADP-ribosyltransferases is a 40-kDa protein associated with the CD8-associated kinase p56^{lck} [124]. In its ADP-ribosylated form, the 40-kDa protein inhibits p56^{lck} kinase activity. It is likely that many more targets for ADP-ribosylation and their physiological relevance remain to be discovered.

Regulation of RT6 action

What is the source of extracellular NAD⁺ for the action of RT6, and how is it controlled? Unlike the situation for extracellular ATP, at the present time there is little evidence for active secretion of NAD⁺. NAD⁺ could come from dead or dying cells, which are common physiological events in the immune system and also in a variety of pathological situations. NAD⁺ can be destroyed by CD38 and PC-1, but not by the “classical ecto-ATPases” such as the CD39 family. Destruction of extracellular NAD⁺ may be an important mechanism for control of ecto-ADP ribosyltransferases by limiting the availability of substrate.

Many covalent regulatory modifications of proteins such as phosphorylation are reversible [125]. It might therefore be anticipated that ecto-ADP-ribosylproteinhydrolases might exist, but so far none have been found. However, some ecto-phosphodiesterases can cleave an AMP-moiety from ADP-ribosylated proteins, leaving a ribose-phosphate unit attached. This mechanism seems to restore the function of ADP-

ribosylated $\alpha 7/\beta 1$ integrin [126]. There is strong evidence that autotaxin works via P1 adenosine receptors [87], but the possibility remains that some of its effects on cellular motility might be due to removal of AMP from ADP-ribosylated membrane proteins [115].

Variation in RT6 genes: functional correlations

In the rat, there is a single RT6 gene. The two known alleles are highly divergent with multiple amino acid substitutions, which probably explains why it is so immunogenic. There are two functional RT6 genes in the mouse. At the present time, the only known RT6-like gene in humans is a pseudogene containing premature stop codons. Given the evidence for the high degree of mobility of the corresponding bacterial genes, it has been suggested that polymorphism of RT6 represents the outcome of a continuing “star wars” battle between host and pathogens [115].

The diabetes-prone BB rat has a defective RT6 gene, and transfusion of RT6⁺ T cells can prevent susceptible rats from developing the disease, suggesting that RT6 may act in some way to regulate autoimmunity [127]. Matthes et al. [128] screened several mouse strains for expression of Rt6-1 and Rt6-2, and found that NZW mice do not express detectable Rt6-2 transcripts, and BXSB mice do not express detectable Rt6-1 transcripts. These mice thus appear to represent naturally occurring knockouts for Rt6-2 and Rt6-1. NZW mice seem to be immunologically normal, suggesting that loss of the Rt6-2 gene is not deleterious, but (NZW $\times$ NWF)F1 and (NZW $\times$ SB)F1 and BXSB mice develop a lupus-like syndrome.

Kanaituka et al. [129] analyzed ARTs designated Rt6-1 and Rt6-2 in BALB/c and C57BL/6 mice. mRNAs both were found in spleen, thymus, and intestinal tissue of both strains, but Rt6-1 mRNA in C57BL/6 mice was detected only at low levels. Sequencing of Rt6-1 cDNA revealed a premature stop codon in C57BL/6 mice, whereas Rt6-2 was normal in both strains. Hence, it would appear that C57BL/6 is a naturally occurring knockout for Rt6-1. The fact that C57BL/6 is generally regarded as immunologically normal suggests that there may be some functional redundancy between the two genes, and makes the correlations between autoimmunity and Rt6 harder to interpret. The fact that humans seem to lack a functional RT6 gene is also puzzling.

The future

ADP-ribosylation is one of the many different ways by which proteins may be posttranslationally modified. In certain cases, notably those associated with bacterial toxins, this modification has profound functional consequences. Functional changes due to the endogenous mammalian ADP-ribosyltransferases seem to be more subtle. The fact that these modifications can take place at the cell surface provides a novel mechanism for the local regulation of cellular activities, but it is still not clear whether the physiologically important targets have yet been identified.

There is a need for antibodies that recognize ADP-ribosylated amino acids. ART-knockout mice may be highly informative. Naturally occurring knockouts might be the cause of human disease. Novel drugs might be developed that interfere with some of these regulatory pathways [115].

5' NUCLEOTIDASE (CD73)

Metabolic roles

5' Nucleotidase (CD73; EC 3.1.3.5) catalyzes the hydrolysis of phosphate groups from the 5' carbon of ribose and deoxyribose portions of nucleotides [15]. It is encoded by a single gene in mammals. The enzyme is a GPI-linked membrane protein that is widely but not ubiquitously expressed. As for most other ecto-enzymes, soluble forms exist [16].

The most obvious role for 5' nucleotidase is in salvage of purines. For example, it hydrolyzes AMP to give adenosine and Pi. This reaction is particularly important because nucleoside phosphates must be converted into the corresponding nucleosides before they can enter cells by facilitated diffusion. This may be important in lymphoid tissues, particularly in the thymus, where massive cell death occurs by apoptosis [110, 111].

Role of 5' nucleotidase in immunodeficiency caused by adenosine deaminase deficiency

In the mouse, CD73 is expressed on subsets of T and B lymphocytes, and expression increases with maturation. Among B cells, expression of CD73 seems to be confined to those cells that have switched isotype. Very few thymic T cells express CD73 in the mouse, although CD73 is prominently expressed on reticular cells of the thymus and peripheral lymphoid organs, suggesting some role in determining the environment of lymphoid cells [130].

There is a strong functional relationship between 5' nucleotidase, which generates adenosine, and adenosine deaminase (ADA), which destroys it by converting it to inosine. This relationship may be important in the pathogenesis of the T cell defect in ADA deficiency [131, 132]. In the human thymus, CD73 expression is low in the cortex, and CD4⁺CD8⁺ T cells are negative for CD73. Even in the medulla, CD4⁺ cells are negative for CD73 and only a minority of CD8⁺ cells are positive for CD73, although medullary stromal cells are strongly positive. ADA expression in the thymus tends to follow a reciprocal pattern, with high expression in the cortex and low expression in the medulla.

Over the years, several different mechanisms have been postulated to explain the selective T cell defect in ADA deficiency on the basis of lack of essential metabolic products or toxicity of metabolites. The vast majority of cells that are born in the thymus die in the thymus by apoptosis as a result of failed selection, and this would be expected to generate an extremely high turnover of nucleotides. Resta and Thompson have recently suggested that the T cell deficiency in ADA deficiency may result from adenosine toxicity in the thymic cortex [131, 132]. ADA degrades adenosine to inosine; adenosine would be expected to build up in ADA deficiency, and a plausible pathway for toxic effects of adenosine on T cells is known. Adenosine can act on A1 receptors to induce apoptosis via a cyclic AMP-mediated pathway, resulting in a block of T cell maturation at the double-negative stage, with failure to rearrange the α chain of the T cell receptor. Somewhat

surprisingly, this block does not seem to affect the numbers of $\gamma\delta$ T cells.

Knockout mice do not seem to have yet been produced for CD73, and it is not known whether they will be viable. Mice lacking ADA die in utero as a result of profound liver toxicity, because the mouse liver seems to be particularly sensitive to the accumulation of ADA substrates [133–135].

5' Nucleotidase in T cell activation: role of the GPI anchor

Ecto-5'-nucleotidase activity has been claimed to be required for generation of alloreactive cytotoxic T cells [136], but this work depended on the selective inhibition of the enzyme by α,β -methyleneadenosine 5-diphosphate, and the validity of the conclusions would depend crucially on whether this drug had any other effects.

Antibodies to several GPI-linked membrane proteins including Thy-1, Ly-6, RT6, and CD73 are capable of inducing T cell activation. The pathways are not well understood, but the presence of a GPI anchor seems to be essential [137–140]. It is interesting that T cell activation by ligation of CD73 in mice requires the *fyn* tyrosine kinase [130], suggesting a possible signaling pathway.

GPI-anchored proteins and glycosphingolipids are localized in membrane microdomains known as “lipid rafts,” and these appear to have specialized properties, including the presence of cytoplasmic signaling molecules such as *src* family kinases, G-proteins, and linker proteins. The lipid portion of GPI anchors is long enough to penetrate to the inner membrane leaflet, raising the possibility of a physical interaction between GPI anchors and these signaling proteins, and it has been speculated that these interactions form the basis for signal transduction via GPI anchors [140]. It would be interesting to determine whether the *fyn* kinase is localized in these rafts.

ALKALINE PHOSPHATASE

Structure and enzymic activity

Alkaline phosphatase (EC 3.1.3.1) was one of the first ecto-enzymes to be discovered [141–143]. The endogenous form is attached to the membrane by a GPI anchor [14, 144], and the widely used water-soluble form arises by cleavage by an endogenous phospholipase during extraction [145].

Alkaline phosphatase has a very broad specificity. It is capable of cleaving almost any phosphomonoester with the release of inorganic phosphate and the corresponding alcohol, phenol, sugar, or other group [141–143]. It can degrade ATP, ADP, and AMP at alkaline pH [143]. Tissue-nonspecific alkaline phosphatase (TNAP) can also cleave pyrophosphate (PPi) to give Pi [146].

Recent data suggest that alkaline phosphatase is part of a superfamily of metalloenzymes that includes phosphopentomutase, cofactor-independent phosphoglycerate mutase, ectophosphodiesterases, and sulfatases. It is likely that they will be found to have similar three-dimensional structure [147].

Genetics and isoenzymes of alkaline phosphatase

There are four “classical” alkaline phosphatase genes encoding the kidney/bone/liver-type (TNAP), placental-type, intestinal-type, and germ-cell-type (or placental variant) enzymes [148]. Recent work has indicated that the level of complexity of alkaline phosphatase genes is even greater than previously thought. A second bovine TNAP gene has been found and several additional genes that are expressed in the bovine intestine have been identified. It seems likely that more will be found in other species [149].

Alkaline phosphatase isoenzymes differ significantly in their stability to heat denaturation, and this is sometimes used in distinguishing between them. They also differ in their catalytic activity [148]. Levamisole is a potent inhibitor of TNAP but not the intestinal form, and has been exploited to reduce the background staining when antibodies conjugated with bovine intestinal alkaline phosphatase are used in immunohistochemistry [150].

The role of alkaline phosphatase in normal physiology is still incompletely understood. Homozygous loss of TNAP in humans gives rise to the rare disease of hypophosphatasia, in which bone structure is grossly disturbed [143]. Knockout mice have been made in which the TNAP gene is disrupted. They are growth-impaired, have epileptic seizures, and die before weaning [151]. They have multiple pathological features, including abnormal bone mineralization, disturbances in intestinal physiology, increased apoptosis in the thymus, and abnormal spleens. Although TNAP does not seem to be essential for the initiation of bone mineralization, it seems to play a role in maintenance of bone mineralization after birth. On the other hand, knockout mice in which the embryonic form of alkaline phosphatase is disrupted seem to develop normally, and show no obvious phenotypic abnormalities [151].

RECEPTORS FOR EXTRACELLULAR NUCLEOSIDES AND NUCLEOTIDES

The effects of ecto-enzymes can be divided into metabolic or regulatory. The metabolic effects of ecto-enzymes on cells can be regarded as generating suitable breakdown products of extracellular compounds so that they can be taken up by cells. Proteins and peptides must be broken down into amino acids, and nucleotides must be converted into nucleosides by removal of their phosphate groups before they can enter cells by facilitated diffusion.

In contrast, most of the regulatory effects of ecto-enzymes are concerned with modulating the effects of extracellular compounds on cell-surface receptors. The classical model is the synapse, where neurotransmitters must be destroyed in order to terminate signaling. A wider role for ecto-enzymes can also be envisaged, where destruction of a pharmacologically active compound occurs at the surface of a non-neuronal cell, either to terminate signaling or to prevent signaling from occurring in the first place. Local ecto-enzymes might also play a role in activating substrates in order to allow them to act on cell-surface receptors.

The literature on the effects of extracellular nucleotides such as ATP and nucleosides such as adenosine on cells has a long history [152, 153]. In spite of this, there was strong resistance to the idea that extracellular nucleotides and nucleosides can also act on cells via specific receptors [154]. This resistance probably arose from skepticism that ubiquitous intracellular metabolic intermediates could mediate highly specialized functions on the external surface of cells.

Although it was established as early as 1959 that ATP could be released upon stimulation of nerves [155], a major obstacle was the lack of plausible mechanisms by which these compounds could be released. Release of ATP from non-neuronal cells was even more difficult to understand. We now know that release of ATP and diadenosine polyphosphates occurs by specific secretory mechanisms, even in non-neuronal cells. In addition, a variety of metabolic stresses ranging from hypoxia to cell death by necrosis or apoptosis will also result in the release of ATP, ADP, NAD⁺, and related compounds [156, 157].

Extracellular nucleotides are now known to have many effects on cells, including neurotransmission, vasodilatation, vasoconstriction, inflammation, induction of apoptosis, and intracellular killing of microbes. The effect depends on the individual cell type, and is commonly modulated by ecto-enzymes.

The actions of nucleotides and nucleosides on cells are mediated by a large family of receptors [154, 158], which are summarized in **Tables 4** and **5**. Purinergic receptors may be divided into those that have a preference for adenosine (P1 receptors) and those with a preference for ATP (P2 receptors). The P2 receptors may be further subdivided into those that act by opening ion channels (ionotropic) and those that act by coupling to intracellular enzymes via G proteins (metabotropic). All currently known P1 receptors are metabotropic. Ionotropic receptors evoke very rapid responses, whereas metabotropic responses are slower.

Receptors for adenosine (P1 receptors)

Receptors for adenosine belong to the “magnificent seven” or serpentine family, which have seven transmembrane segments. The amino terminus is outside the cell, and the carboxy terminus is intracellular. Signaling is coupled to intracellular enzymes through G proteins, which can act in a stimulatory or inhibitory manner [158, 159]. At this time, adenosine receptors are subdivided into four groups, A₁, A_{2A}, A_{2B}, and A₃ (Table 4).

The physiological responses to adenosine are best known in the central nervous system and the cardiovascular system, but effects are seen in cells throughout the body. The first were documented by Drury and Szent-Györgi in 1929 [152], and included negative inotropy and chronotropy, which are now known to be mediated by A₁ receptors.

Adenosine has also been implicated in the induction of sleepiness [160], and there is strong evidence that the effects of caffeine and other xanthine drugs are mediated by blockade of adenosine receptors, probably A₁ and A_{2A} [161]. Amounts of caffeine that are consistent with three to six cups of coffee per day result in a detectable rise in plasma adenosine levels, by an unknown mechanism [162]. Xanthines are also commonly used as bronchodilators for treatment of asthma, and it is likely that these effects are mediated via blockade of adenosine receptors.

Mice in which the A_{2A} receptor has been disrupted by homologous recombination are fertile and normal at a gross level, but seem to be more anxious and aggressive than wild-type mice. They also have higher blood pressure and their platelets are more easily aggregated [163, 164].

Apoptosis can be induced in some cells by stimulation of adenosine receptors [157]. As mentioned earlier, this has been postulated to be a pathogenic mechanism in the generation of T cell deficiency seen in ADA deficiency [131]. There is strong pharmacological evidence that the effects of autotaxin on motility of melanoma cells is mediated via A₁ adenosine receptors, and the motility responses to adenosine can be transferred to Chinese hamster ovary cells by transfection with A₁ receptor cDNA [131].

Receptors for ATP, ADP, diadenosine polyphosphates, and related nucleotides (P2 receptors)

P2 purinoceptors can be subdivided into P2X, which are ionotropic and have two transmembrane segments, and P2Y, which are metabotropic G protein-coupled receptors with the classical seven-transmembrane-segment architecture. Their properties are summarized in Table 5.

A detailed discussion of P2 receptors is beyond the scope of this review, and the reader is referred to the recent review of Abbracchio and Burnstock for further information [8]. In addition to their effects on muscle and nerve, P2 receptors can mediate an enormous range of physiological processes, including cell death by both apoptosis and necrosis [157]. They can profoundly influence cell proliferation, inflammation, injury due to reperfusion and ischemia, wound healing, and development and resorption of bone.

Of particular interest for the readers of this journal are the P2X₇ receptors (previously classified as P2Z), which are expressed on macrophages and a variety of other hemopoietic cells [165]. P2X₇ receptors respond to agonist binding by the opening within milliseconds of a channel for small cations, and then within seconds by the opening of a much larger pore that allows penetration of molecules up to about 900 Da, including propidium dyes.

P2X₇ receptors mediate apoptosis and intracellular killing, and seem to play an important role in inflammation, phagocytosis, and tissue repair. They induce apoptosis in macrophages, and in so doing, cause the macrophages to kill intracellular mycobacteria [166]. This effect cannot be mediated by cytokines alone, and seems to require the presence of extracellular ATP. It appears to be mediated by mechanisms that are distinct from reactive nitrogen or oxygen intermediates, and may provide important new insights into defense mechanisms against tuberculosis. There is some evidence that CD8⁺ cytotoxic T cells can secrete ATP, which may provoke the killing of intracellular organisms [167]. Extracellular ATP might also result in cytotoxicity by mechanisms that are independent of fas and perforins.

ECTO-PEPTIDASES

The second major class of ecto-enzymes includes peptidases and transpeptidases (**Table 6**).

TABLE 4. Selected Properties of G-Protein-Coupled Receptors for Adenosine (P1 Receptors)

Subtype	Signaling mechanisms	Selected biological actions
A ₁	Inhibits adenylyl cyclase. Stimulates guanylate cyclase. Stimulates phospholipases A ₂ and C. Activates K ⁺ channels. Inactivates Ca ²⁺ channels. Modulates glucose transporters.	Affinity for adenosine in nanomolar range. Expressed in many localized areas of the brain, notably cortex, cerebellum, hippocampus, and thalamus, as well as heart and vascular smooth muscle, adipocytes, testis, bladder, and eye. Induction of sleepiness (antagonized by xanthine drugs such as caffeine). Depression of neurotransmission, sleep induction, antinociception, ethanol-induced motor incoordination, bronchoconstriction, sodium retention, negative chronotropic and inotropic effects on heart. Inhibition of lipolysis, stimulation of glucose uptake in white fat cells. Superoxide generation. Mast cell activation. Cell motility. Negative role in lung surfactant secretion; possible role in adult respiratory distress syndrome (ARDS) and acute renal failure.
A _{2A}	Stimulates adenylyl cyclase.	Affinity for adenosine in high nanomolar range. Expressed in brain in striatum, nucleus accumbens, and olfactory tubercle. Also expressed in heart, lung, liver, some non-neural cells, blood vessels, platelets, fibroblasts, and thymus. Facilitation of neurotransmission. Antagonizes dopamine receptors. Vasodilatation. Inhibition of platelet aggregation. Adhesion of polymorphs to vascular endothelium. Mice with germline deletion are more anxious and aggressive than wild type and have higher blood pressure; and platelets are more efficiently aggregated.
A _{2B}	Stimulates adenylyl cyclase. Can also signal through phospholipase C and/or ion fluxes.	Widespread expression, notably colon, bladder, brain, spinal cord, and lung. Affinity for adenosine in low micromolar range. Enhancement of degranulation of mast cells with release of IL-8.
A ₃	Negatively coupled to adenylyl cyclase. Can also signal through phospholipase C and/or ion fluxes.	Widespread distribution, notably on sperm, mast cells, lung, kidney, heart, cerebral cortex, striatum, and olfactory bulb. Enhancement of degranulation of mast cells. Inactivation of eosinophil migration.

For further details, see Ref [159].

Peptidases are not boring. They are key players in the regulation of extracellular processes such as activation of complement and blood clotting, as well as intracellular processes including the induction of apoptosis via a cascade of caspases. Ecto-peptidases can be expected to have a similar

range of functions, some of which are already known, while many others remain to be discovered [2, 3].

Ecto-peptidases are mostly type II membrane proteins with short amino-terminal cytoplasmic tails, single transmembrane regions, and large extracellular catalytic domains. They are

TABLE 5. Selected Properties of Receptors for ATP, ADP, and Related Compounds (P2 receptors)

Subtype	Signaling mechanism	Selected biological actions
P2X P2Z (P2X ₇).	Binding of ATP opens cation-selective channel. See P2X.	Mostly confined to muscle and neural tissue. Vasoconstriction. P2Z is expressed on hemopoietic cells, including lymphocytes, macrophages, and mast cells. It is now known to belong to the P2X family (P2X ₇). Like other P2X members, it opens a ligand-gated cation channel, but this channel undergoes a time-dependent transition to a larger “pore” that allows passage of molecules up to 0.9 kDa. P2X ₇ can induce apoptosis and killing of intracellular mycobacteria in macrophages.
P2Y	7-Transmembrane receptors. Most P2Y receptors signal through the phosphoinositide pathway and release intracellular calcium. P2Y ₁₁ is unique in that it is coupled to adenylyl cyclase without inducing mobilization of calcium stores.	Widely distributed in various tissues, including hemopoietic cells. P2Y ₁₁ can induce vasodilatation and differentiation in hemopoietic cells.
P2U	See P2Y.	P2U receptors are now known to be part of the P2Y family (P2Y ₂).
“P2T”		Platelet aggregation. P2T is expressed on platelets, but is not yet structurally characterized. It may be identical to P2Y ₁ .

TABLE 6. Ecto-peptidases and Transpeptidases of Leukocytes and Other Cells

Catalytic activity	CD number, aliases, abbreviations	Selected biological functions
Carboxypeptidase M	MAX.1/MAX.11	Activation, inactivation, and change of specificity of cytokines?
Aminopeptidase A (EC 3.4.11.7)	Glutamyl aminopeptidase, BP-1, 6C3, EAP, APA	Conversion of angiotensin II to angiotensin III
Neutral endopeptidase (EC 3.4.24.11)	CD10, CALLA (common acute lymphoblastic leukemia antigen; enkephalinase; neprilysin)	Activation, inactivation, and change of specificity of cytokines?
Aminopeptidase N (EC 3.4.11.2)	CD13	Cleavage of cytokines; trimming of peptides bound to class II MHC. Possible role in cell adhesion.
Dipeptidyl peptidase IV (EC 3.4.14.5)	CD26, thymocyte-activating molecule (THAM)	T cell activation; activation, inactivation, and change of specificity of cytokines. Cell adhesion. Carrier for ADA in human but not in mouse. Restraint of melanoma progression?
Angiotensin-converting enzyme (EC 3.4.15.1)	CD143 (dipeptidyl carboxypeptidase)	Conversion of angiotensin I to angiotensin II
γ -Glutamyl transpeptidase (EC 2.3.2.2)		Recycling of intracellular glutathione; possible role in control of nitric oxide production.

often expressed on the apical surface of epithelia, notably in the gut, where they take part in the digestion of luminal proteins and polypeptides before their absorption.

In contrast to their constitutive expression on epithelia, ecto-peptidases of leukocytes tend to be developmentally regulated, although they are rarely confined to a particular lineage. It is widely believed that they act as local regulators of cellular function by cleavage of cytokines or cell-surface receptors. As is the case for ecto-nucleotidases, the most difficult issue is the identification of physiologically relevant substrates.

There are striking changes in expression of ecto-peptidases in malignant cells [168]. In some cases, malignant transformation is associated with a marked increase in ecto-protease expression, whereas in others it is associated with down-regulation. There is substantial evidence that these changes are functionally important and not merely epiphenomena. They

seem to depend on the individual cell type. Many of these effects are probably mediated via cleavage of autocrine or paracrine polypeptides, which affect cell growth and differentiation [2, 3]. Expression of membrane proteases is also associated with invasiveness, probably by direct effects on the extracellular matrix but possibly also via effects on cytokines [169].

CD10 (neutral endopeptidase; EC 3.4.24.11)

CD10 is a zinc-binding metalloprotease that cleaves peptide bonds on the amino side of hydrophobic amino acids. It is also known as neprilysin, enkephalinase, and CALLA (common acute lymphocytic leukemia antigen). It is a homodimeric type II membrane protein (subunit molecular weight 100,000), with many features in common with other membrane peptidases including CD13, CD26, and 6C3/BP1. It is expressed at high levels on the brush border of gut and kidney, and is widely used as a marker for acute lymphocytic leukemias, which are most commonly pre-B in phenotype. In the bone marrow, CD10 is restricted to lymphoid precursors and mature granulocytes. In the thymus, CD10 is expressed on stromal cells that bind CD4⁺CD8⁻ T cells.

CD10 has a very broad peptidase specificity. It can cleave enkephalins, f-Met-Leu-Phe, substance P, bradykinin, bombesin-like peptides, atrial natriuretic factor, endothelin, oxytocin, and angiotensins I and II [99]. However, at this time there is no evidence that CD10 can degrade peptides involved in the growth or maturation of lymphoid precursors.

Expression of CD10 is lost or reduced in several malignancies, notably in the development of androgen-independent forms of prostate cancer [170] and small cell carcinoma of the lung. CD10 inhibits growth of small cell carcinoma of the lung by cleavage of bombesin-like autocrine growth factor peptides [171].

CD10 knockout mice are normal at the gross level, but are more sensitive to endotoxic shock than their wild-type littermates [172]. One possible mechanism for this hypersensitivity might be that CD10 can inactivate biologically active peptides, and deletion of CD10 might be expected to strengthen and prolong their actions. It was of interest that CD10 knockout mice had increased sensitivity to tumor necrosis factor and interleukin-1, suggesting that the sites of action of CD10 are downstream from these mediators.

CD13 (aminopeptidase N; EC 3.4.11.2)

Like CD10, CD13 is a homodimeric type II membrane protein. The subunits have a molecular weight of about 150,000–170,000. It is related in sequence to the ecto-peptidase BP-1/6C3 (see below). It is expressed on the brush border of gut and kidney. Within the hemopoietic system, CD13 has been considered a myeloid cell marker. It is expressed on stem cells and all stages of myeloid development, granulocytes, monocytes and their precursors, and most acute myeloid leukemias. However, CD13 is also present on lymphoid precursors and some acute lymphocytic leukemia cells, but is not present on normal mature lymphocytes. It is also expressed on endothelial cells, fibroblasts, osteoclasts, bone marrow stromal cells, and bile canaliculae [2, 173].

CD13 is a zinc-binding metalloprotease of broad specificity, and is a member of the family known as gluzincins [173]. Its

catalytic activity resembles that of thermolysin, which is the only member of the family to have been crystallized. It can cleave many different substrates, including enkephalins and other endogenous opioids, tuftsin, and the chemotactic peptides IL-8 and fMet-Leu-Phe. It has also been implicated in the trimming of peptides in the groove of class II MHC molecules.

Expression of CD13 is prominent at sites of inflammation, such as joints in rheumatoid arthritis, where it may degrade bradykinins and enkephalins in conjunction with CD10 and CD26. It is up-regulated in many cell types by IL-4 and interferon- γ , and can be induced on lymphocytes by contact with other cells that already express CD13 [173].

There is evidence that CD13 plays a role in cell-cell adhesion. When cultured melanoma cells form colonies, CD13 is found mainly at sites of cell-cell contact, and it seems to be associated with the extracellular matrix. It has been suggested that focal adhesion kinase (FAK) might be a meeting point between adhesion and peptidase substrates, playing a role in peptidase-mediated effects on adhesion, motility, and anchorage-independent growth [168, 173].

CD13 has been subverted by viruses. It is the cellular receptor for transmissible gastroenteritis virus in pigs and coronavirus 229E, which causes upper respiratory tract infections in humans (**Table 7**). These activities do not seem to require its proteolytic activity [173].

BP-1/6C3 (glutamyl aminopeptidase, EC 3.4.11.7)

Glutamyl aminopeptidase (EAP) is an exopeptidase that cleaves at acidic residues from the amino terminus of polypeptides [174]. It has a preference for glutamate (E) rather than aspartate (D). It has sometimes been referred to as angiotensinase, because it converts angiotensin II to angiotensin III, which has diminished pressor activity but which may be biologically

active in the central nervous system. It is also known as aminopeptidase A (APA).

BP-1/6C3 is a disulfide-bonded homodimeric type II membrane protein and is related to CD13 in sequence. Subunit molecular weight is about 160,000. It is expressed on the brush border of gut and the proximal convoluted tubule of kidney as well as renal glomeruli, vascular endothelium, and bile canaliculi.

Within hemopoietic cells, BP-1/6C3 is restricted to immature B cells. It is up-regulated by IL-7 and viral transformation, and was initially thought to be associated with malignant transformation of B cells, but later work has not confirmed this idea [175]. It is not expressed in mature B cells, memory B cells, or antibody-secreting cells, and it therefore forms a useful marker for early B cell precursors.

Mice that lack functional BP-1/6C3 appear normal in their development, and there is no evidence of disturbances to either B or T cell differentiation [174, 176].

CD26 (dipeptidyl peptidase IV, EC 3.4.14.5)

CD26 represents one of the most perplexing of the ecto-enzymes because of its multitude of functions [177]. It is a membrane protease, a T cell-stimulating molecule, a carrier for the intracellular enzyme adenosine deaminase, and may also play a role in cell-cell adhesion by binding to fibronectin and collagen [178, 179]. It is impossible to do justice to CD26 in the space available, and the reader is referred to recent reviews for more detailed information [168, 180, 181].

CD26 is expressed on epithelial cells of intestine, the proximal convoluted tubule in the kidney, bile canaliculi, and in the prostate. Within the hemopoietic system, it is expressed on mature thymocytes and T cells, and is up-regulated on T cell activation. As is typical for ecto-enzymes, CD26 is a type II noncovalent homodimer. Its enzyme activity is unusual in that it cleaves dipeptides from the amino terminus of proteins if proline or alanine are present as the second amino acid in the intact protein.

The enzymic activity of CD26 is responsible for the intestinal absorption of proline-containing polypeptides. The Fischer 344 strain of rat lacks functional CD26 due to failure to transport it to the cell surface, and has severely disturbed renal absorption of proline-containing peptides [182]. These rats also show altered response to some peptide hormones, but do not seem to have any gross immunological abnormalities [180]. As far as I am aware, knockout mice for CD26 have not yet been produced.

There are several lines of evidence implicating CD26 in T cell activation. Antibodies to CD26 can enhance the activation of T cells to suboptimal stimuli, and can inhibit mitogenic or antigen-specific responses. The effects seem to require cross-linking of the CD26 molecule. Soluble CD26 can also enhance the *in vitro* antigen-specific proliferation of T cells in response to tetanus toxoid. Although the mechanism of enhancement is not known, this activity requires proteolytic activity [183].

The cytoplasmic tail of CD26 is only six amino acids in length, and any signaling effects are likely to be indirect, either via its enzymic activity or via associated proteins such as the tyrosine phosphatase CD45, which has the ability to influence

TABLE 7. Non-Enzymic Functions of Ecto-enzymes

Ecto-enzyme	Function	Is enzymic activity required?
CD13	Receptor for transmissible gastroenteritis virus in pigs	No
	Receptor for human coronavirus 229E (upper respiratory tract infections)	No
	Cell-cell adhesion	?
CD26	Binding site for adenosine deaminase (humans, not mice)	No
	Cell-cell adhesion	No
	T cell activation	Data contradictory
CD38	B cell activation	?
	Cell-cell adhesion	?
CD73	T cell activation	No
	Cell-cell adhesion (further evidence required)	?
PC-1 (E-NPP1)	Resistance to insulin; binds to insulin receptor	No
Autotaxin (PD-1 α ; E-NPP2)	Stimulation of cell motility	Yes

the proximal parts of the cascade of intracellular signaling from the T cell receptor [180, 181, 184].

One of the most enigmatic properties of CD26 is the fact that it binds the cytoplasmic enzyme ADA and displays it on the lymphocyte surface [185], although the association occurs in humans but not in mice [180, 181]. The pathway by which a cytoplasmic enzyme can find its way onto the cell surface bound to a membrane protein is currently unknown. Although its full meaning is still unclear, the association of ADA and CD26 suggests a potential functional linkage between the immunological functions of CD26 and the severe immunodeficiency seen in ADA deficiency [131, 180, 181].

Ecto-peptidase-mediated changes in specificity of cytokines

Even quite minor changes in the amino-terminal region of cytokines can sometimes make large differences to their biological activities. Truncation of the cytokine RANTES (regulated on activation, normal T cell-expressed and secreted) by CD26 changes the specificity of receptor interactions and target cells, and this might be a physiologically significant control mechanism [186–188].

Similarly, deletion of the amino-terminal amino acid of the monocyte chemotactic protein-1 converts it from an activator of basophil mediator release to an eosinophil attractant [189]. Whether this cleavage occurs *in vivo* or is purely a laboratory curiosity remains uncertain, but in principle, it could be mediated by aminopeptidase N (CD13).

Role of CD26 in malignancy

Marked changes in the expression of CD26 occur in malignancy [168]. CD26 is not expressed in the normal thyroid, but is present at high levels in follicular, papillary, and Hürthle cell carcinomas of the thyroid. Increased CD26 expression is also correlated with greater aggressiveness in T cell lymphomas.

In view of these findings, it is paradoxical that CD26 expression is lost during the transformation of melanocytes into malignant melanoma. Recent work suggests that this loss may play a causative role in the development of the malignant phenotype [168, 190].

When CD26 is transfected into melanoma cells under the control of a tetracycline promoter, induction of synthesis of the enzyme was able to reverse the block in differentiation. It also caused reversion to a more normal phenotype, with loss of tumorigenicity and anchorage-independent growth, and acquisition of dependence on exogenous growth factors. It is surprising that the suppression of tumorigenicity and reversal of the block in differentiation required enzymic activity, whereas dependence on exogenous growth factors did not, suggesting that the effects of CD26 on these activities are mediated by distinct and separable mechanisms.

These experiments were particularly impressive because they seemed to demonstrate causality rather than mere association. A key question is how the proteolytic activity of CD26 reverses the malignant phenotype. Is it by proteolytic activation of an endogenous or autocrine inhibitory polypeptide, or are other mechanisms at work?

In summary, CD26 remains a puzzle. Although its sequence

is highly conserved, its distribution and biological effects vary considerably between species. The fact that the Fischer 344 rat lacks functional surface CD26 and yet does not seem to have major immunological abnormalities could mean that CD26 is not essential for T cell activation, or that its function might be able to be replaced by another molecule, or that the pathways of T cell activation differ significantly between species. The biological meaning of the surface association with ADA also remains unclear. The dramatic effects of loss and restoration of expression of CD26 in melanoma cells point to novel mechanisms of control of normal growth and differentiation as well as tumorigenesis.

ECTO- γ -GLUTAMYL TRANSPEPTIDASE

The ectoenzyme γ -glutamyl transpeptidase (GGT) catalyzes the hydrolysis of γ -glutamyl peptide bonds and transfers the glutamyl group to a suitable acceptor. A major substrate is glutathione (GSH), which is the predominant intracellular reducing agent, and may be present in cells at concentrations of up to 10 mM [191]. Cleavage of GSH by GGT is the first step in the process by which extracellular GSH, which cannot enter cells, is recycled into glutamate, cysteine, and glycine, which can. GGT is found on the surface of many cell types, notably the proximal convoluted tubule of the kidney, liver, small intestine, mammary gland, and choroid plexus. It is also found on the surface of human T and B lymphocytes, and its expression increases on activation. GGT may also be involved in the control of nitric oxide production in T cells [192]. Knockout mice for GGT are retarded in growth and have markedly elevated plasma and urinary GSH, and intracellular concentrations of GSH are about 50% of normal [193]. They show increased excretion of methylmercury, a compound that forms a covalent adduct with glutathione [194].

ECTO-PROTEIN KINASES

Intracellular protein kinases regulate numerous cellular activities by adding phosphate groups to the hydroxyl-containing amino acids serine, threonine, or tyrosine. They are now extremely well characterized at the molecular level, and constitute a very large and diverse family [195, 196]. If it could be proven that similar control mechanisms exist outside cells, it would be a most exciting and important advance. There have been numerous reports of ecto-protein kinases, but these have been somewhat controversial [197]. Difficulties include contamination with cytoplasmic kinases, metabolic interconversions involving phosphate, cell viability, and leakage across damaged membranes. There has also been skepticism concerning whether ATP is really secreted by cells. The intracellular ATP concentration is typically about 5 mM, and leakage from damaged or dead cells would be enough to cause significant artifacts. However, numerous physiological mechanisms for release of ATP from cells have now been documented (see below).

Redegeld et al. [197] have summarized the criteria for detection of ecto-protein kinase activity. Highly viable cells

must be used, and the extracellular substrates must not penetrate the cell membrane. Low concentrations of ATP should be used, and incubation times kept short. Addition of millimolar concentrations of inorganic phosphate should not affect phosphorylation, and experiments should be carried out in serum-free medium, because serum may contain protein kinases. The sites of addition of phosphate to proteins must be shown to be located on their extracellular domains. It has also been suggested that the phosphorylated region should be able to be released from the cell by proteolysis, but this requirement may be difficult to meet because many membrane proteins are quite resistant to proteolytic removal from cells.

After these strict criteria are applied, a considerable body of evidence remains in support of the existence of ecto-kinases [197–200]. Ecto-kinases appear to be capable of phosphorylating the ecto-domains of certain membrane proteins, including the T cell receptor for antigen [199] and the β -amyloid precursor protein [201]. Other proteins that appear to be phosphorylated by ecto-kinases include the collagen and thrombospondin receptor CD36, N-CAM, and a 48-kDa/50-kDa protein duplex that is associated with maintenance of hippocampal long-term potentiation [200].

Numerous secreted proteins also appear to be phosphorylated by ecto-kinases, including basic fibroblast growth factor, vitronectin, fibronectin, complement components, atrial natriuretic peptide, osteopontin, and bone sialoprotein [197]. In many cases, these phosphorylation events are associated with physiological changes, although a causal link has not been established in all cases.

The evidence for phosphorylation of secreted proteins and ecto-domains of membrane proteins is convincing. Information concerning the enzymes responsible for these phosphorylation events is much less definitive. An “atypical protein kinase C” has been claimed to be present on the surface of neurons in the brain, on the basis of inhibition of phosphorylation and biological effects by the IgM monoclonal antibody 1.9, which was raised against intracellular protein kinase C and inhibits its activity. It was presumed that this antibody cross-reacts with an atypical ecto-protein kinase C that is related to intracellular protein kinase C. At this time there is no direct molecular evidence to support this proposal, and the mechanism of inhibition of these activities and the molecular target for this antibody (apart from intracellular protein kinase C itself) are not yet known.

The epitope recognized by monoclonal antibody 1.9 was detectable on the surface of platelets by immunofluorescence [202]. Preliminary results using immunoelectron microscopy suggested that the relevant antigen was detectable in the junctional region of synaptic clefts [200], but there does not seem to be any further characterization in the literature. IgM antibodies are notorious for their promiscuous cross-reactivity [203], and definitive proof of the existence of “ecto-protein kinase C” is still lacking.

A casein kinase-like protein has been described on the surface of T cells. It is blocked by the inhibitor K-252b which has been widely used to study ecto-kinases [197]. This compound, which has been shown to block ecto-phosphorylation but not intracellular phosphorylation, inhibits the activity

of cytotoxic T lymphocytes, suggesting a role for ecto-kinases in T cell-mediated immune responses. It also inhibits synapse formation between cultured cortical neurons [197]. However, questions have been raised concerning whether K-252b is truly membrane impermeant [204].

One of the most detailed attempts to characterize ecto-protein kinases at the molecular level was made by Walter et al. [205]. Two activities, named ecto-PK I and ecto-PK II were identified. Ecto-PK I consisted of a single 40-kDa moiety, whereas ecto-PK II had catalytic subunits of 43 and 40 kDa and a third noncatalytic subunit of 28 kDa. The molecular nature of ecto-PK I was not identified, but ecto-PK II cross-reacted with antibodies to the well-known protein intracellular kinase CK II. Peptide mapping and mass spectrometry suggested that “ecto-PK II” was similar to, or identical with CK II. The question of whether this activity was truly an “ecto-kinase” or merely contamination by cytoplasmic CK II was not definitively resolved.

The plasma cell membrane glycoprotein PC-1 (ecto-phosphodiesterase I; E-NPP1; PDNPI; NPP γ) has been claimed to be a threonine-specific ecto-kinase that is capable of phosphorylating itself as well as other proteins [49–52]. Although PC-1 has been characterized in great detail at the molecular level, the evidence for its involvement in phosphorylation of proteins other than itself is weak.

Culp et al. [42] identified the active site residue of bovine intestinal phosphodiesterase (a protein closely related to PC-1) as threonine, on the basis that it could be labeled by thymidine 5'-[α - 32 P]triphosphate. They postulated that covalent phosphorylation of this residue was part of the catalytic cycle. The corresponding threonine in PC-1 is amino acid 238 (corrected numbering system). Belli et al. [53] showed that recombinant PC-1 could be labeled by adenosine 5'-[α - 32 P]triphosphate and also by adenosine 5'-[α - 32 P]triphosphate. They also showed that mutagenesis of threonine 238 to serine, tyrosine, or alanine abolished both labeling by adenosine 5'-[α - 32 P]triphosphate and phosphodiesterase activity. These authors were not able to confirm claims that PC-1 was capable of phosphorylating proteins other than itself, and proposed that the phosphorylation seen by other workers was due to contaminating kinases.

Subsequent work by Bollen's group [54] showed that ATP hydrolysis and autophosphorylation by PC-1 were two distinct catalytic reactions. It was shown that autophosphorylation of PC-1 was associated with inactivation of its phosphodiesterase/pyrophosphatase activity, and proposed that autophosphorylation of PC-1 serves as an autoregulatory mechanism that makes threonine 238 unavailable for cleavage of extracellular nucleotides when they become scarce. They also showed that the corresponding threonine residue on bovine intestinal phosphodiesterase could not be autophosphorylated, demonstrating that autophosphorylation is not an intrinsic property of the nucleotide pyrophosphatase reaction. PC-1 appeared to be capable of phosphorylating histone IIa, although this protein is an extremely poor substrate, with a K_m of $>100 \mu\text{M}$ [52]. It was argued that it is not necessary for a protein kinase to possess a protein-kinase consensus sequence because a member of the phosphatidylinositol 3-kinase family is also a *bona fide* protein

kinase, even though it lacks a conventional protein kinase catalytic domain [195, 196, 206].

Early work suggested that autophosphorylation of PC-1 was stimulated by nanomolar concentrations of acidic fibroblast growth factor (aFGF) [49]. Later, Uriarte et al. found that aFGF inhibited the phosphodiesterase activity of PC-1 [52], but this was subsequently shown to be due to a non-protein contaminant in commercial preparations (probably EDTA) [207]. Pure aFGF neither stimulated nor inhibited PC-1 [52].

From this brief discussion, it will be apparent that much more work needs to be done before ecto-protein kinases can be regarded as well-defined entities. Cloning and molecular characterization of ecto-kinases would be a major advance.

UNIFYING CONCEPTS

Sources and fate of extracellular ATP

Extracellular ATP can no longer be regarded as exclusively an artifact due to leakage from damaged or dead cells. There is overwhelming evidence that ATP is released physiologically as a neurotransmitter (often together with other compounds such as noradrenaline) (207a), by release of granules from platelets and cells of the adrenal medulla, and possibly via P glycoprotein. In addition, ATP may be released under certain pathological situations including inflammation and exposure to lipopolysaccharide, hypotonic cellular environment, and hypoxia [197].

Extracellular ATP is pharmacologically active via several different pathways, including direct action on P2 purinergic receptors and indirectly via conversion to ADP, AMP, and adenosine and subsequent interaction with P1 purinergic receptors. In addition, the phosphate moiety of ATP may be transferred to proteins via ecto-protein kinases [197], and may form complexes with calcium in both normal bone and in pathological calcification [70, 71] (Fig. 4).

Grouping into families based on sequence and three-dimensional structure

The cloning and sequencing of cDNAs encoding ecto-enzymes and membrane receptors has had enormous impact on the thinking about their function. In many cases, the availability of a sequence has prompted the reclassification of a receptor or enzyme. Taxonomy based on sequence, and therefore on evolutionary history, seems to provide a more satisfactory approach than taxonomy based on function. Separation of

members of the CD39 family from those of the PC-1 family makes sense, even though both families are ATPases. Similarly, the grouping of the various purinergic receptors based on sequence and evolutionary history has a certain logic.

This is not to imply that function is unimportant. Structure and function should be seen as a two-way street. Sequences have often provided important functional insights, and substantial progress will only be made by integration of function with structure [208].

There can be no doubt that the currently known families will grow in size. It seems likely that in many cases where a gene seems to have no relatives, future work will reveal that it is part of a new or existing family. When the sequence homology is very low, family relationships may be subtle and not immediately obvious, but the folding pattern may be conserved long after any obvious sequence homology has been lost. Traces of a common ancestry may be first revealed by a common three-dimensional structure. This was the case for the ADP-ribosyltransferases, where the relatedness between the bacterial and mammalian enzymes only became apparent after the crystal structures were solved.

New algorithms are being developed to detect these more distant sequence relationships, and these may allow detection of common folding patterns. The discovery of previously unsuspected relationships in this way may facilitate predictions and determinations of three-dimensional structure. This approach has revealed that alkaline phosphatase and ecto-phosphodiesterases are part of a superfamily of metalloenzymes that includes phosphopentomutase and cofactor-independent phosphoglycerate mutase and sulfatases [147]. Because the structures of some of these are known, the discovery of these relationships may facilitate the solving of the structures of other family members.

What are the physiological substrates for ecto-enzymes?

We know a great deal about what ecto-enzymes can do. What is less clear is whether it is physiologically important. In most cases, we can only guess at the truly relevant *in vivo* substrates. By what criteria can we decide that a substrate is physiologically relevant?

Although it is a truism to say that the substrate must be available for the enzyme, it is not enough to demonstrate potential availability. The substrate must be available in situations that make biological sense. Proving this point is far from trivial.

ATP is certainly available, both as an actively secreted product and from activated, stressed, dying, or dead cells. The situation for NAD⁺ is much less clear, but we should not exclude the products of dying or dead cells from consideration because cell death is often a physiological process, especially in the immune system. Even when cell death is a manifestation of pathology, evolution has produced defense mechanisms to limit the damage, to initiate repair, and to recycle products of the corpse.

There is good evidence for the physiological role for the ectoenzyme γ -glutamyl transpeptidase. The enzyme provides a plausible mechanism for recycling glutathione, and when it is

Fig. 4. Metabolic fate of extracellular ATP.

deleted by homologous recombination, glutathione recycling is impaired and the mice have retarded growth [193]. There is also strong evidence for similar roles for the nucleotidases CD38 and CD73 [110].

Interaction of ecto-enzymes and receptors: activation, inactivation, and change of specificity for substrate

Some of the ways that ecto-enzymes can interact with purinergic mechanisms are shown in Figure 3. Ecto-enzymes may terminate signaling, alter the specificity of signaling, or eliminate unwanted signals. They may also create a signaling molecule from a precursor.

In vivo, the functional specificity of a given receptor depends on the rate of destruction of ligand as much as the intrinsic specificity of the receptor itself. Interpretation of some of the older pharmacological experiments involving whole tissues might therefore need to be reevaluated. Experiments in which cloned receptor cDNAs are transfected into cells with known ecto-enzyme properties will give more accurate information about the intrinsic specificity of the receptor, but *in vivo* experiments will remain essential to determine functional specificity in its biological setting.

A receptor may be rendered functionally inactive in a cell if a local enzyme destroys its ligand, and this might represent a useful control mechanism. In evolutionary terms, this is just as valid an evolutionary strategy as turning off the gene. It has been used by nature to impose selective mineralocorticoid responsiveness by cells that also possess the receptor for glucocorticoids [10–12], and there is every reason to think that it might apply in other systems.

Ecto-enzymes have the potential to change the type of a response by altering ligands such that they bind to different types of receptor, possibly even on different cell types, as seen in the CD26-mediated truncation of RANTES [168].

Detailed understanding of the *in vivo* physiology will require parallel information concerning the subtype of receptors expressed in a tissue or cell, and concomitant information about the local expression of all relevant ecto-enzymes. This is quite a tall order, and will require a much larger range of specific antibodies than are currently available.

Tissue distribution

It is common for the tissue distribution of ecto-enzymes to vary between species. This is the case for members of the PC-1 family of ecto-phosphodiesterases, and also for ecto-5' nucleotidase and CD38. The meaning of these differences is rarely obvious. Is it possible that expression is conserved in the tissues or cells where it is important, but is allowed to vary in those in which it does not matter? Could the differences in distribution contribute to speciation?

There are numerous examples where an enzyme is widely expressed, and yet when it is deleted, the pathology is confined to a subset of tissues or cells, as occurs with ecto-phosphodiesterase PC-1, where the major pathology is in bone and cartilage [26, 27]. Adenosine deaminase is also very widely expressed, yet the pathology caused by its deletion is confined to the

immune system in humans, although it also causes severe liver damage in mice [133].

Is it possible that expression of a given gene in a given tissue may sometimes be purely an accidental consequence of evolutionary tinkering with combinations of transcriptional factors, and be of no functional significance at all? If so, how can we understand function?

Function depends on location

Ecto-enzymes may have different functions in different locations [173]. Proteases or nucleotidases on the brush border of gut or kidney most likely have a purely nutritional function, but when expressed in lymphoid tissues they might have a more subtle role in regulating the cytokine environment. They may act as molecular switches, just like individual transistors in a computer, where identical units perform different functions depending on their context.

Multifunctionality

Many ecto-enzymes have multiple functions, and sometimes these functions do not seem to be directly related to each other or to enzymic activity (**Table 7**). For several ecto-enzymes, there is evidence for a role in cell activation (CD26, CD38, CD73, RT6), cell adhesion (CD26, CD38, and CD73), or binding to an apparently unrelated protein (binding of ADA to CD26). Some cell-surface enzymes have been subverted to act as receptors for viruses, notably CD13.

The evidence for the multifarious functions of ecto-enzymes is not equally strong. This is especially true for some of the claimed adhesion functions of some enzymes. The *in vitro* systems used to demonstrate activation of lymphocytes by antibodies to ecto-enzymes are highly artificial. It is often unclear whether they have any real significance *in vivo* [see ref. 140].

Although some skepticism regarding *in vivo* significance of multifunctionality is probably in order, there is ample precedent that it really does occur in nature, at least in some systems [209]. Evolution works by tinkering, adding new layers of mechanisms on top of the old.

What can phylogenetic comparisons tell us?

Although the tissue and cellular distribution of ecto-enzymes often varies between species, their amino acid sequences are usually highly conserved. Sequence conservation is generally regarded as reflecting functional constraints, and implies a biological penalty in the form of disease or reduced fitness if the gene is allowed to drift. Catalytic domains tend to be particularly conserved, and their sequences can often be used to identify new members of a family (see Fig. 2).

Lack of sequence conservation might be interpreted in several different ways. It could imply that the gene is not important, or that another gene can take its place, or that it has diverged to a new function.

A third possibility is that conservation of the sequence of the same gene between species may be patchy, even within a given protein. Islands of conservation may reflect functionally important domains. Functional constraints probably explain the presence of islands of conservation between the cytoplasmic

tails of mouse and human PC-1 (Table 3). It may be speculated that these islands contain intracellular targeting sequences that mediate basolateral location in polarized epithelial cells; this hypothesis has the virtue of being testable.

How can we make a definitive attack on the problem of causality?

The field of ecto-enzymes is plagued by the problem of how to distinguish associations from causative roles. Finding an association is easy; proving that it really means something is hard.

One of the most powerful ways to resolve this dilemma is to use engineered or natural mutations to delete or modify genes, and to apply the complementary approach of inserting genes. Convincing evidence that the autotaxin-mediated motility response is mediated via production of adenosine acting on adenosine receptors was obtained by transfer of responsiveness via cloned A1 adenosine receptor cDNA [87]. Evidence for a causal role for the loss of CD26 in the progression of malignant melanoma was greatly strengthened by the demonstration that the abnormal phenotype could be reversed by transfection with functional CD26, and that the phenotype was only reversed on induction of CD26 synthesis [168, 190].

Naturally occurring or manmade knockout mice have been very useful in determining what is really important. Sometimes the phenotype is dramatic and illuminating. Other times there has been no obvious phenotype, or the phenotype is unexpected and confusing, as might be the case if a given gene did different things in different tissues. The next generation of knockout mice, in which the knockout is inducible and tissue-specific, promises to be even more powerful.

The synapse revisited

Until recently, the synapse was regarded as being exclusively within the domain of neurons. However, there is now a growing body of evidence to suggest that mechanisms that are closely related to those occurring in synapses may mediate local communication between other cells [210, 211].

The synapse has three essential components; a source of transmitter, a receptor for the transmitter, and an enzyme that degrades the transmitter to terminate signaling (**Fig. 5A**). Many ecto-enzymic systems discussed in this review can be considered to be part of this triad. Communication between non-neuronal cells might also be possible by a process that is analogous to the synapse (**Fig. 5B**).

THE FUTURE

In the next few years, synergistic interactions between physiology, pharmacology, pathology, and molecular genetics will bring substantial progress. Many of the areas that are now confusing and incoherent will be clarified, but progress toward identification of the physiologically relevant substrates for ecto-enzymes will probably remain slow. The study of ecto-enzymes will greatly improve our understanding of normal physiology and disease, and may yield novel drugs and therapeutic interventions.

Fig. 5. Similarities between the synapse and purinergic signaling in non-neuronal cells. (A) Neuronal synapse, consisting of source of neurotransmitter (1), receptor for neurotransmitter (2), and transmitter-destroying enzyme. (B) Modulation of non-neuronal cells by nucleotides or similar molecules. The source of transmitter (1) may be from another cell or possibly autocrine. The transmitter acts on a specific receptor (2), and the responding cell has a transmitter-destroying enzyme (3).

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