1. Introduction

1.1. CARBONIC ANHYDRASES

1.1.1. Carbonic Anhydrase Metalloenzymes

Carbonic anhydrases (CA; carbonate dehydratases) are Zn^{2+}-binding metalloenzymes that catalyze the reversible hydration of carbon dioxide to carbonic acid (Meldrum and Roughton, 1933; van Goor, 1948):

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons HCO_3^- + H^+$$

These enzymes are found in animals, plants and some bacteria. While α-carbonic anhydrases are primarily found in animals, the β- and γ-carbonic anhydrases are typical for plants and prokaryotes (Tashian, 1989). Mammalian carbonic anhydrases participate in a variety of physiological processes like respiration, bone resorption, gluconeogenesis, renal acidification, formation of cerebrospinal fluid and gastric acid (Sly and Hu, 1996; Hewett-Emmett and Tashian, 1996; Tureci *et al*., 1998; Ivanov *et al*., 1998). Carbonic anhydrase isozymes are highly polymorphic, differing in kinetic properties, subcellular localization, tissue distribution and susceptibility to inhibitors.

To date, eleven enzymatically active members of an α-carbonic anhydrase family have been identified in humans. In addition, carbonic anhydrase-related proteins (CA-RPs) have been described (for review Chegwidden, 2000). To the CA-RPs belong CA VIII, CA X, CA XI, CA XIII and receptor protein tyrosine phosphatase-β and -γ. These are acatalytic isoforms due to the substitution of at least one of three histidine residues that are required to bind Zn^{2+}. The striking conservation of their sequences among different species suggests that they might retain important cellular functions. One of the examples is receptor protein tyrosine phosphatase-β (RPTP-β). The physiological role of RPTP-β has been connected to the modulation of cell interactions and to developmental processes of the nervous system, where its N-terminal, extracellular CA-like domain plays a central role (Sakurai *et al*., 1997; Peles *et al*., 1995).

1.1.2. Physiological Function of Carbonic Anhydrases

The expression pattern of active CAs is very variable. CA I is highly expressed in erythrocytes and in spite of its low catalytic activity, it complements CA II in red blood cells by 50%. Although CA I is the most abundant non-hemoglobin protein in erythrocytes, its reported absence does not cause any hematological disorder in human (Sly and Hu, 1995). Cytosolic CA II is one of the most efficient catalysts in biological systems and is expressed in practically all organs. It is so far the only CA whose deficiency has been directly associated with human disease. Patients affected with this recessively inherited syndrome suffer from osteopetrosis with renal tubular acidosis and cerebral calcification (Sly *et al*., 1983). Additionally, CA II is abundantly expressed in some brain tumors and in gastric and pancreatic malignancies (Parkkila *et al*., 1995a; Parkkila *et al*., 1995b). CA III is a very low activity isozyme and is mostly found in skeletal muscles (Lloyd *et al.*, 1985). CA IV is a cell surface isozyme bound to the plasma membrane by the glycosylphosphatidylinositol anchor. In kidney, CA IV is localized on the apical membrane of epithelial cells where it facilitates reabsorption of bicarbonate. In addition, a contribution of CA IV to the physiology of colon, brain, lung and heart is assumed from its high expression in these organs and its very high enzymatic activity (Okuyama *et al.*, 1992; Sly and Hu, 1995). Two isoforms of mitochondrial CA V (CA VA and CA VB) have been identified. Both CA VA and CA VB share high sequence homology. Similarly to CA VA, CA VB appears to be a low activity isozyme (Fujikawa-Adachi *et al*., 1999a). So far, CA VI seems to be the only secreted member of the CA family. It is expressed in salivary glands and is a protein component of the enamel pellicle (a thin layer of proteins covering the teeth). Although CA VI has considerably lower enzymatic activity, functionally, it might be an important regulator of pH homeostasis on dental and gastroesophageal epithelial surfaces (Aldred *et al.*, 1991; Kivela *et al.*, 1999). The physiological function of cytosolic CA VII is still unclear despite its good kinetic properties (Montgomery *et al.*, 1991; Earnhardt *et al.*, 1998). CA IX and CA XII are transmembrane proteins that are associated with some malignancies and might be functionally related to oncogenesis (Zavada *et al*., 1997; Ivanov *et al*., 1998; Tureci *et al*., 1998). CA IX will be extensively discussed later. *CA12* mRNA is normally expressed in kidney, colon, prostate, testis, lung, brain, pancreas, ovary and is overexpressed in renal tumors (Ivanov *et al*., 1998; Tureci *et al*., 1998). CA XIV is the latest reported human isozyme abundant in the central nervous system (CNS) (Mori *et al*., 1999). Its role in the

CNS is so far unknown. Significant expression of *CA14* mRNA was also shown in heart, skeletal muscles and in some parts of gastrointestinal tract (Fujikawa-Adachi *et al.*, 1999b).

The enzyme activity, structure and cellular localization relations among the active α-carbonic anhydrases are shown in figure 1.

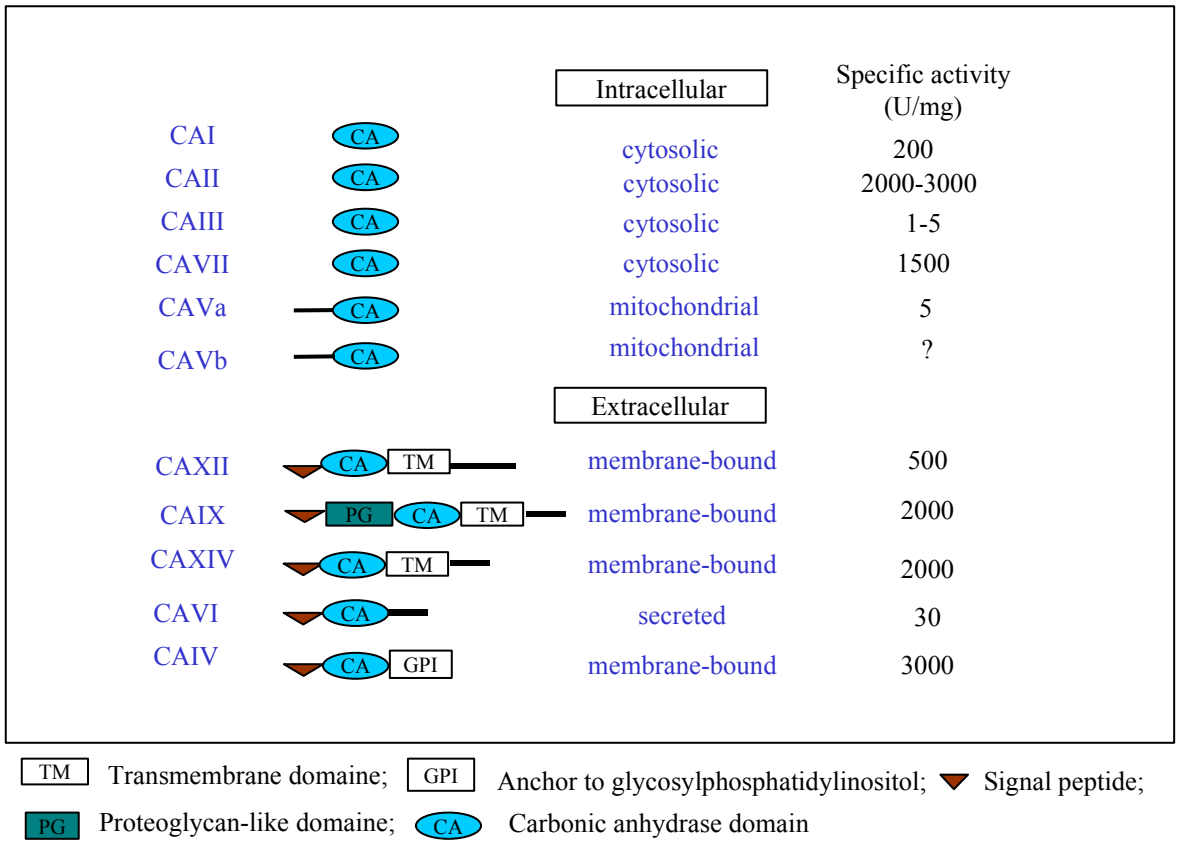

Fig. 1. Structural, activity and cellular localization relations among the active α-carbonic anhydrases (Mori *et al.*, 1999; modified; Karhumaa *et al.*, 2000; modified).

1.1.3. Animal Models of Carbonic Anhydrase Deficiencies

To understand the physiological importance of the carbonic anhydrases, the logical approach would be to construct animal models with targeted mutations. Unfortunately, to date, this has not been accomplished for any of the carbonic anhydrase isozymes.

Spontaneous null mutations of CA I in humans as well as in animals were registered. A common homozygous naturally occurring mutation of CA I was described for the pigtail macaque. However, this monkey completely lacking CA I does not exhibit any disease, as is also true in humans (Sly and Hu, 1995).

Heritable mutations of CA II were induced in mouse by chemical mutagenesis using N-ethyl-N-nitrosourea (ENU) (Lewis *et al.*, 1988). Substitution of C by T resulted in the introduction of an early stop codon at position Gln155. Similar to human, CA II deficient mice exhibited a defect in renal acidification, impairment in growth and in late onset calcification of blood vessels. Unlike in humans with CA II deficiency syndrome, the cerebral calcification and typical osteopetrosis was not observed in CA II mutant mouse. However, this mouse model was used also in epilepsy research where it was demonstrated that CA II plays an important role in the susceptibility to seizures although the mechanism remains unclear (Velisek *et al*., 1993).

1.1.4. Association of Carbonic Anhydrases with Cancers

The knowledge about the mechanism of cancer development and the surrounding conducting factors, important for its fast spread, has increased exponentially during the last few decades. Since the late 20ties of the 20th century it has been known that the extracellular pH of tumor cells is on average more acidic than that of the normal cells (Warburg, 1926; Griffiths, 1991). Martinez-Zaguilan *et al*. (1998) reported distinct regulatory capacity of intracellular pH in poorly and highly metastatic human melanoma cells. The invasiveness of cancer cells could be suppressed *in vitro* by acetazolamide, a potent CA inhibitor (Parkkila *et al*., 2000).

The exact role of CA activity in cancer has not been determined yet. It is possible the aberrant control of expression of certain CAs in malignant cells facilitates their growth or/and enhances their invasive behavior through regulation of the extracellular pH (Ivanov *et al*., 1998).

The expression of some carbonic anhydrases in tumor lesions has been studied in detail (Ivanov *et al*., 1998; Parkkila *et al*., 1995a; Parkkila *et al*., 1995a; Wykoff *et al*., 2000). As it was mentioned above, the cytoplasmatic CA II was found to be more abundant in some tumors of the nervous system and carcinomas of the stomach and pancreas. Although the results were consistent, CA II did not prove restrictive enough to be useful for diagnostic screening.

Recently, CA XII was shown to be related with von Hippel-Lindau carcinogenesis (Ivanov *et al*., 1998). It was isolated from large-scale screen of genes differentially expressed in a renal cell carcinoma (RCC) cell line with mutated von Hippel-Lindau tumor suppressor gene (VHL) before and after transfection of the wild type VHL. Furthermore,

CA12 mRNA expression levels were found to be up-regulated in 10% of patients with RCC in comparison to surrounding normal kidney tissue (Tureci *et al*., 1998). In colorectal tumors, localization of CA XII seems to display a characteristic diffuse pattern and the immunoreactivity increases with the degree of dysplasia (Kivela *et al.*, 2000).

So far the most studied CA, that has been clearly related to oncogenesis, is CA IX.

1.2. CARBONIC ANHYDRASE IX (MN)

1.2.1. MN protein alias CA IX

The human CA IX (originally named MN protein) was first identified in the cervical carcinoma cell line HeLa using monoclonal antibody M75 (Pastorekova *et al*., 1992). Cloning and sequence analysis of a cDNA coding for MN revealed that the protein contains a large extracellular carbonic anhydrase domain. Thus, it was later classified as a new member of the carbonic anhydrase family and designated as MN/CA IX protein (Pastorek *et al*., 1994; Hewett-Emmett and Tashian, 1996).

It has been proposed that MN/CA IX protein may participate in acid-base balance, intercellular communication and cell proliferation. All the initial findings supported the notion that it might be a novel type of (proto)oncogene. Characteristically, MN/CA IX is abnormally expressed in various human carcinomas originating from MN/CA IX-negative tissues, but is absent or diminished in tumors derived from MN/CA IX-positive ones. Normally, its expression is restricted to only few tissues, especially to mucosa of the alimentary tract.

1.2.2. Characteristic Features of MN/CA IX Protein

MN/CA IX is a transmembrane protein localized on the cell surface where it forms oligomers linked by disulfide bonds (Pastorekova *et al*., 1992; Zavada *et al*., 1993). The immature form of the protein was found in the cytoplasm (Pastorekova, personal communication).

On Western blotting, MN/CA IX protein is detectable by a monoclonal antibody (M75) as a twin band of 54/58kDa. The protein is N-glycosylated with a carbohydrate chain of 3-kDa size. Under non-reducing conditions, it is able to form homo-oligomers (most likely trimers) with a molecular weight of 153kDa (Pastorekova *et al*., 1992; Zavada *et al*., 1993, Zavada *et al*., 2000). The cell surface position and oligomerization appear to be the

prerequisites for the biological activity of MN/CA IX (Pastorekova, personal communication). Figure 2 shows the hypothetical two-dimensional model of the MN/CA IX protein and its situation on the surface of the cell-membrane.

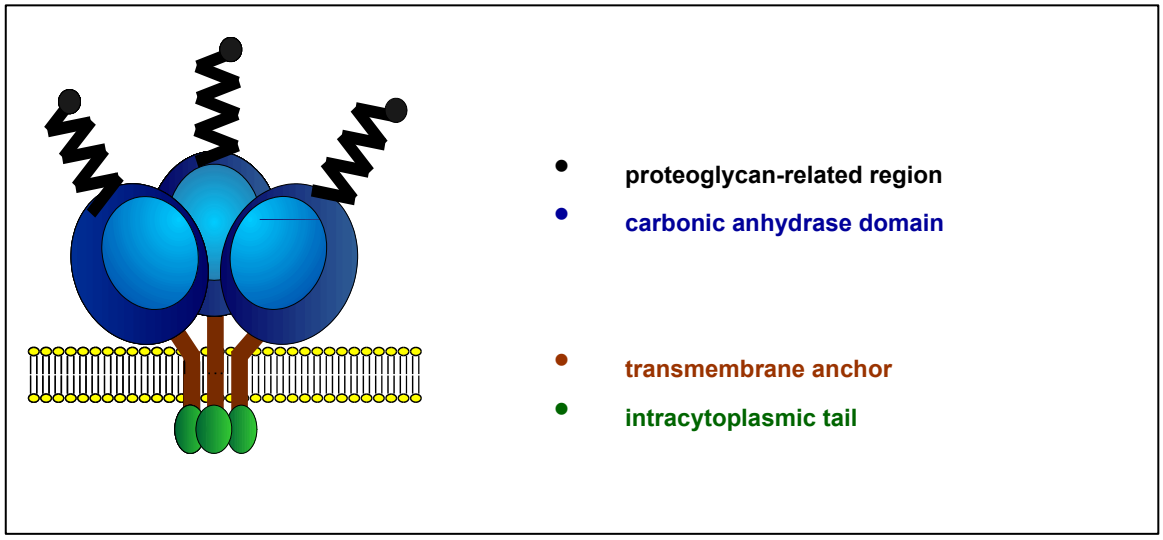

Fig. 2. Two dimensional model of human MN/CA IX protein as a transmembrane trimer anchored in the cytoplasmatic membrane (with a kind permission of Dr. S. Pastorekova).

1.2.3. Physiological Expression of MN/CA IX *Protein*

Expression of human MN/CA IX protein under physiological conditions is restricted to epithelial cells of the gastrointestinal (GI) tract, where it is expressed in a basolateral manner (Pastorekova *et al*., 1997, Saarnio *et al*., 1998a). The protein is most abundant in mucosa of the stomach. All major cell types of the gastric epithelium, including pit cells, zymogenic cells and parietal cells, are positive for MN/CA IX (Tab. 1).

In the intestinal epithelium, regional expression of MN/CA IX gradually decreases along the cranial-caudal axis of the gut (Saarnio *et al*., 1998a). The most intense expression is in the duodenum and jejunum, while the ileum, caecum, and ascending colon exhibit just moderate staining levels. Sporadic expression was seen in the distal colon. In contrast to the stomach epithelium that contains MN/CA IX in all major cell types, the protein expression in the intestinal epithelium is restricted to the enterocytes of the crypts. These cells are endowed with the highest proliferative activity. As they migrate along the intestinal villus, they differentiate and coincidentally, they lose the expression of MN/CA IX. In a recent study of Saarnio *et al*. (1998b), MN/CA IX was shown to be co-expressed with a proliferation marker Ki-67. Based on this study, MN/CA IX was proposed to serve as a potential marker of proliferation in healthy and pre-malignant colorectal mucosa. In addition

to stomach and intestine, MN/CA IX protein expression was detected in the gallbladder epithelium and in the pancreatic duct (Pastorekova *et al*., 1997).

A similar pattern as described in human was also found in the rat GI tract. The major difference was noticed in the intestine, where the expression was strong in the superficial part of the mucosa. In contrast to human, no protein was detected in the pancreatic duct (Pastorekova *et al*., 1997).

Organs	Histological site	Rat	Human
Oral mucosa	Surface epithelial cells	ND	-
Parotid gland	Serous cells	-	-
	Duct cells	-	-
Submandibular	Serous cells	-	-
Gland	Mucous cells	-	-
	Duct cells	-	-
Esophagus	Surface epithelial cells	ND	-
	Mucous glands	ND	-
Stomach	Surface epithelial cells	+++	+++
	Parietal cells	+++	+++
	Zymogen cells	+++	+++
Duodenum	Surface epithelial cells	++	ND
	Brunner´s glands	-	ND
Jejunum	Surface nongoblet epithelial cells	-	ND
	Goblet cells	-	ND
Ileum	Surface nongoblet epithelial cells	-	+
	Goblet cells	-	+
Colon (proximal)	Surface nongoblet epithelial cells	+++	+
	Goblet cells	+++	+
Colon (middle)	Surface nongoblet epithelial cells	++	+
	Goblet cells	++	+
Colon (distal)	Surface nongoblet epithelial cells	+	ND
	Goblet cells	+	ND
Liver	Hepatocytes	-	-
	Duct cells	+	++
Gallbladder	Luminal epithelial cells	ND	+++
Pancreas	Zymogen cells	-	-
	Islets of Langerhans	-	-
	Duct cells	-	+

Tab. 1. Distribution of MN/CA IX protein in the human and rat alimentary tracts. –, no staining; +, weak staining: ++, moderate staining: +++, intense staining; ND, not done (Pastorekova *et al*., 1997).

1.2.4. Association of MN/CA IX *Protein with Oncogenesis*

The association between MN/CA IX expression and oncogenesis was first suggested by observation of the MN/CA IX relationship with the tumorigenic phenotype of HeLa x fibroblast hybrid cells constructed by Stanbridge *et al*. (1981; 1982). In non-tumorigenic hybrid cells (CGL1) MN/CA IX was absent, but it was strongly expressed in the tumorigenic segregants (CGL3). Moreover, Zavada *et al*. (1993) reported expression of MN/CA IX in some human neoplastic clinical specimens and several tumor cell cultures, and its absence in corresponding healthy tissues.

Transfection of the NIH3T3 cell line with *MN/CA9* cDNA (Pastorek *et al*., 1994; Zavada *et al*., 1997) led to a transformed morphology, increased saturation density and loss of anchorage dependence as evidenced by the ability of cells to grow in soft agar.

These facts triggered profound studies that resulted in the recognition of MN/CA IX as a new tumor-associated antigen. The data showed that it may prove useful in the early diagnosis of several types of human tumors. Liao *et al*. (1994) found significant expression levels of MN/CA IX in about 90% of tissue specimens of all histological categories of cervical carcinomas including adenocarcinoma, squamous cell carcinoma, and adenosquamous cell carcinoma. These findings were confirmed in other studies where MN/CA IX was proposed as an early biomarker of cervical neoplasia (Costa *et al.*, 1995; Brewer *et al.*, 1996; Liao and Stanbridge, 2000). Abnormal MN/CA IX expression was associated also with carcinomas of the esophagus (Turner *et al.*, 1997), lung (Vermylen *et al.*, 1999), kidney (Liao *et al.*, 1997; McKiernan *et al.*, 1999) and colon (Saarnio *et al*., 1998b).

Interestingly, in malignant stomach specimens and in several cell lines derived from stomach carcinoma, the expression of MN/CA IX is considerably decreased or missing (Pastorekova *et al*., 1997). This suggests that MN/CA IX may function as a putative negative regulator of normal gastric cell proliferation.

The significance of MN/CA IX in oncogenesis still remains unclear. There is evidence that the protein possesses adhesion properties, as it is able to mediate adhesion of cells to a non-adhesive solid support *in vitro* (Zavada *et al*., 2000). These adhesion properties might be involved in aberrant cell-cell or cell-matrix interactions (Lieskovska *et al*., 1999). However, it also cannot be excluded, that via the carbonic anhydrase activity MN/CA IX contributes to an acidic extracellular microenviroment facilitating the tumor growth and invasion (Ivanov *et al.*, 1998).

1.2.5. Antigen for MAb G250 is Identical to MN/CA IX

In 1986 Oosterwijk *et al.* described a monoclonal antibody G250 (MAb G250) that has been raised against a human renal cell carcinoma (RCC). MAb G250 reacted with 90% of primary RCC and 82% of metastatic RCC lesions but not with the surrounding, healthy renal tissue. The cross-reactivity with normal tissues was restricted to the gastric mucosa and the bile duct (Oosterwijk *et al.*, 1986; Oosterwijk *et al.*, 1993). Recently, the cDNA coding for the G250 antigen was cloned, and sequence analysis revealed identity to MN/CA IX antigen (Grabmaier, 2000).

Several clinical studies showed excellent visualization of RCC primary tumors and of metastatic lesion with ^{131}I - labeled MAb G250. This method was able to reveal tumor lesions that could not be identified by X-ray or computer tomography (CT) (Steffens *et al*., 1997; Steffens *et al*., 1999b). The circulating *MN/CA9/G250* mRNA was detectable already in peripheral blood of RCC patients by reverse transcriptase polymerase chain reaction (RT - PCR) (Uemura *et al.*, 1999).

For potential use of the mouse MAb G250 in radioimmunotherapy a chimeric antibody was constructed (cG250) where the constant regions of the mouse antibody were substituted by their human homologues. The tumor uptake of ^{131}I - labeled cG250 (as well as of ^{131}I - labeled MAb G250) in RCC patients was so far the highest reported for solid tumors. However, the distribution of the antibody was highly heterogeneous. This fact may limit the efficacy of the radiotherapy (Steffens *et al*., 1999a; Oosterwijk *et al.*, 1993). Using an other approach, bi-specific MAbs (G250/anti-CD3 or G250/anti-CD55) were constructed to enhance specific lysis of the tumor cells either by activation of the complement or by antibody-dependent cell mediated cytotoxicity (ADCC) (Luiten *et al.*, 1996; Blok *et al.*, 1998). In both cases the results suggested that the bi-specific MAbs may improve the immunotherapeutic treatment of RCC malignancies.

The results of these authors suggest MN/CA IX/G250 is the best marker for clear cell RCC and it could also be used as a potential therapeutic target.

1.2.6. Sequence analysis of MN/CA9 cDNA

The human *MN/CA9* cDNA was cloned and sequenced by Pastorek *et al*. (1994) and subsequently the genomic structure was characterized by Opavsky *et al*. (1996). No differences in nucleotide sequence have been found between cDNA isolated from gastric mucosa and the original cDNA derived from HeLa cells. That indicates that cDNA

mutations are not associated with abnormal protein expression in tumor tissues (Pastorekova *et al*., 1997). The *MN/CA9* cDNA has a single splicing variant and the open reading frame is coding for protein sequence of 459 amino acids (aa) with predicted molecular weight of 49,7kDa. Based on the deduced amino acid sequence MN/CA IX protein is composed of an N-terminal signal peptide (aa 1-37), an extracellular part (aa 38-414), a transmembrane anchor (aa 415-434) and a short intracytoplasmatic C terminus. The extracellular part of the mature protein consists of proteoglycan-like (PG-like) region (aa 53-111) and a carbonic anhydrase (CA) domain (aa 135-391) (Fig. 3).

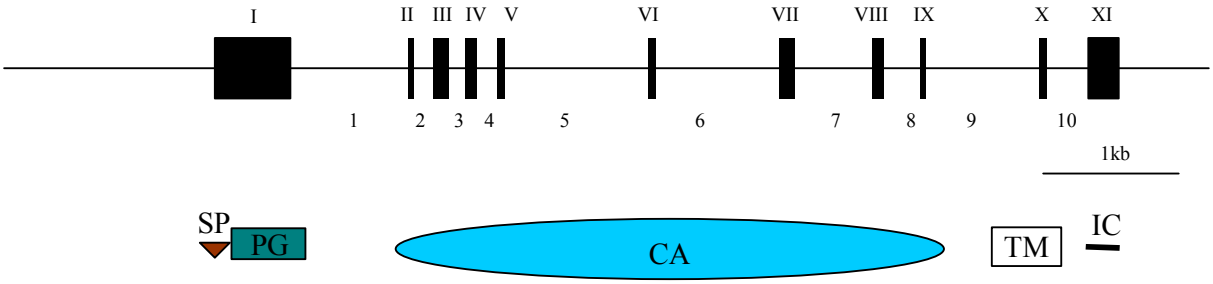

Fig. 3. The Exon-intron situation of the human *MN/CA9* gene. The exons are numbered by roman numbers and the introns by arabic numbers. The scale bar belongs to the genomic map. MN/CA IX protein-domains - SP - signal peptide, PG - proteoglycan like domain, CA - carbonic anhydrase domain, TM - transmembrane anchor, IC - intracytoplasmic tail, are beneath the coding sequence of the exons (Opavsky *et al*., 1996; modified).

The sequence corresponding to the PG-like region has some similarities with a large proteoglycan - aggrecan. There is about 38% homology between the keratan sulfate attachment domain of aggrecan and the PG-like domain of MN/CA IX (Opavsky *et al*., 1996). It is still not known whether the glycosaminoglycan chain is attached to MN/CA IX, respectively whether MN/CA IX belongs to the proteoglycan family.

However, it was recently shown that the PG-like region is responsible for the adhesion capacity of the protein and might be involved in the proposed role of MN/CA IX in cell-cell communication and cell-matrix adhesion (Zavada *et al*., 2000).

The presence of PG-like domain is the feature distinguishing MN/CA IX from the other known CAs. It is assumed that MN/CA IX isozyme might be involved in acid-base balance in the stomach where its expression is most abundant and where it is exposed to variable pH conditions. The CA domain of MN/CA IX behaves as a highly active enzyme with the highest proton transfer rate (Silverman, 2000; Fig. 1). It is not surprising since all five residues (His 94, His 96, His 119, Glu 106 and Thr 199 (employing the mature human CA I nomenclature in which position 1 is the first residue after the N-terminal Met

(Hewett-Emmett and Tashian, 1996)) important to form the Zn^{2+} binding core and essential for fully active carbonic anhydrase, are conserved in MN/CA IX. This property facilitated purification of MN/CA IX by adsorption to sulfonamide-agarose columns and by elution with acetazolamide (Zavada *et al*., 2000). This method is applicable only for enzymatically active carbonic anhydrases that bind sulfonamides.

MN/CA IX seems to be most closely related to the secreted salivary CA VI. Together with CA IV, CA VI, CA XII and CA XIV it also shares the identical position of two cysteine residues that form an internal disulfide bond.

1.2.7. The Genomic Structure of MN/CA9 Gene

MN/CA9 gene belongs, together with *CA6* and membrane-bound *CA4* genes, to a closely related group in evolution that represents the oldest mammalian CA genes (Jiang and Gupta, 1999). The tumor associated *CA12* and *CA14* probably also belong to this evolutionary oldest group of CAs. Their extracellular CA domains are closely related to CA IX and CA VI but the genomic sequences are so far not known.

MN/CA9 gene is thought to have evolved by gene shuffling. The location of *MN/CA9* was mapped to the chromosome band 9p12-p13 (Nakagawa *et al.*, 1998). The gene is organized in eleven exons and ten introns (Opavsky *et al*., 1996). The first exon is coding for the signal peptide and the PG-like domain. The CA domain is encoded by exons 2 - 8, the short transmembrane anchor by exon 10 and the cytoplasmatic tail by exon 11 (Fig 3).

The regulatory region of *MN/CA9* gene contains a TATA-less promoter. Although it possesses 59% of GC residues it does not exhibit additional features of typical GC-rich promoters of housekeeping genes (Opavsky *et al*., 1996, Kaluz *et al*.,1999).

The fragment -173/+31 (position relative to the transcription initiation site) was identified as a minimal promoter of *MN/CA9* gene. It contains cis elements that are responsible for the MN/CA IX characteristic expression pattern. Five protected regions (PR) that bind transcription factors were identified in this fragment by DNase I footprinting. One of them (PR4 -134/-110) exhibits silencing activity. Two protected regions, PR1 and PR2, bind activating factors, while the contribution of PR3 and PR5 to transcriptional regulation is diminished. The promoter appears to operate in a complex manner via synergy between silencing and activating mechanisms (Kaluz *et al*., 1999).

There is some evidence that the tumor suppressor protein p53 exerts repression activity on MN/CA IX expression (Kaluzova *et al*., 2000). P53 is considered one of the

pivotal actors in carcinogenesis. Using the CAT reporter assay the *MN/CA9* promoter was silenced in co-transfection experiments by the wt p53 in a dose-dependent manner. In agreement with the co-transfection experiment up-regulation of endogenous p53 severely influenced the activity of the promoter construct and had a partial repressing effect on the protein expression.

The von Hippel-Lindau (VHL) protein, another member of the tumor suppressor family, has been shown to down-regulate MN/CA IX protein together with CA XII in renal carcinoma cell lines (Ivanov *et al*., 1998). VHL is mutated in most sporadic clear cell renal carcinomas and in hereditary VHL disease that predispose affected individuals to a variety of tumors. The transfection of wtVHL into VHLmut/MN/CA IX+ cells led to abrogation of MN/CA IX expression and strongly diminished its promoter activity (Grabmaier, 2000; Ivanov *et al*., 1998). These research results were followed by the latest study of Wykoff *et al*. (2000) reporting insensitivity of VHLmut/ MN/CA IX+ cells to hypoxia, what could be reconstituted by wtVHL expression. Furthermore this group showed direct positive impact of hypoxia on MN/CA IX expression in many other tumor cell lines and tissue extracts from investigated tumors. It was shown that the *MN/CA9* minimal promoter contains the hypoxia response element (HRE) that binds the transcription complex hypoxia inducible factor-1 (HIF) response element and *MN/CA9* was proved to be a new HIF target gene. Among these genes some were already identified for which the tumor growth promoting function was well established.

The methylation status of DNA is an important regulator in neoplastic incidence. Recently it was shown that the activation of *MN/CA9* gene in renal carcinoma cell lines was associated with hypomethylation of its 5' region (Cho *et al*., 2000).

Despite significant progress in the last couple of years, interplay among described regulatory mechanisms and their individual contribution to an overall expression pattern of MN/CA IX under different conditions has not been clarified.

1.2.8. MN/CA IX *Protein is an Adhesion Molecule*

Cell-matrix and cell-cell interactions play critical roles during many physiological and pathological processes including morphogenesis and tumorigenesis. It is still not clear why MN/CA IX is aberrantly expressed in a great variety of carcinomas and how it is engaged in carcinogenesis on one hand and in the development of the GI tract on the other one. The expression level of MN/CA IX in carcinoma cells increases with higher density of cell

culture and following the loss of anchorage during the growth in suspension. These facts together with the strategic localization of MN/CA IX protein on the cell surface indicated its role in cell-cell communication. Recent studies suggest that MN/CA IX functions as an adhesion molecule that facilitates loss of contact inhibition and anchorage independence (Pastorek *et al*., 1994; Zavada *et al*., 1997; Zavadova *et al*., 1997; Lieskovska *et al*., 1999). However, the precise mechanism of its action is not known.

A lot of effort has concentrated on the localization of the region critical for adhesion capacity of MN/CA IX. *In vitro* adhesion assay using affinity purified MN/CA IX and synthetic PhD-library derived oligopeptides revealed that the adhesion properties reside in the PG-like region. Interestingly, monoclonal antibody M75 was able to abrogate cell attachment to MN/CA IX suggesting that its epitope overlaps with the adhesion-mediating site of MN/CA IX protein (Zavada *et al*., 2000).

Also, it cannot be excluded that the CA domain of MN/CA IX protein might be engaged in adhesion. Receptor-type protein tyrosine phosphatase (RPTP-β), a protein distantly related to MN/CA IX, contains both a carbonic anhydrase-like (CA-like) domain, proteoglycan domain (PG) in its extracellular region. The two distinct domains (CA-like and PG domain) bind to different neuronal receptors and through these interactions control the growth of neural cells. While the PG domain can bind to neurones and inhibit neurite outgrowth, the CA-like domain binds to neuronal contactin and induces neurite growth and differentiation (Sakurai *et al*., 1997). Although the CA domain of RPTP-β is not endowed with enzymatic activity, two of three Zn^{2+} binding His are still conserved. Presumably its highly packed hydrophobic core, as well as the hydrophobic residues exposed on the surface of CA structure, might be involved in protein-protein interactions (Peles *et al*., 1995). Similarly, the CA domain of MN/CA IX forms a high and deep hydrophobic pocket that is structurally predisposed to serve as a receptor site.

1.3. DEVELOPMENT OF THE MOUSE STOMACH AND ARCHITECTURE OF THE GASTRIC MUCOSA

1.3.1. Development of the Gastrointestinal Tract in the Mouse

During the embryonic development, the gastrointestinal (GI) tract of the mouse derives from different primary germ layers (Gilbert, 1988). Mesoderm is involved in formation of connective tissue and musculature. A single layer of proliferative endoderm that is detectable around embryonic day 7 (E7), develops in few days into a pseudostratified

epithelium of primitive gut (Karam, 1999; Ramalho-Santos *et al.*, 2000). The embryonic endoderm creates the linings of the digestive tube. The buds of the digestive tube form the liver, gallbladder and pancreas. The pharynx is a chamber in the anterior region shared by respiratory and digestive tubes. Posterior to the pharynx the digestive tube constricts to the esophagus lined by several layers of epithelial cells. The esophagus is followed by the stomach that develops as a dilated region of the digestive tube (around embryonic day E12). The intestine is formed more caudally. The lumen of the stomach and the intestine is covered by a single layer of epithelial cells and its surface area is increased by many evaginating and/or invaginating epithelial units (Karam, 1999).

1.3.2. The Anatomy of the Stomach

The main function of the stomach is to process and transport food. After feeding, the contractile activity of the stomach helps to mix, grind and evacuate the food in small portions of chyme into the small bowel.

The stomach is a large dorsoventrally flattened sac located in the left anterior quadrant of the abdominal cavity (Gilbert, 1988). Anatomically, the stomach can be divided into forestomach, body of the stomach (corpus), and constricted pyloric antrum, that marks the boundary between stomach and small intestine. The lesser and greater curvatures give to the body of the stomach its characteristic anatomic shape (Fig. 4).

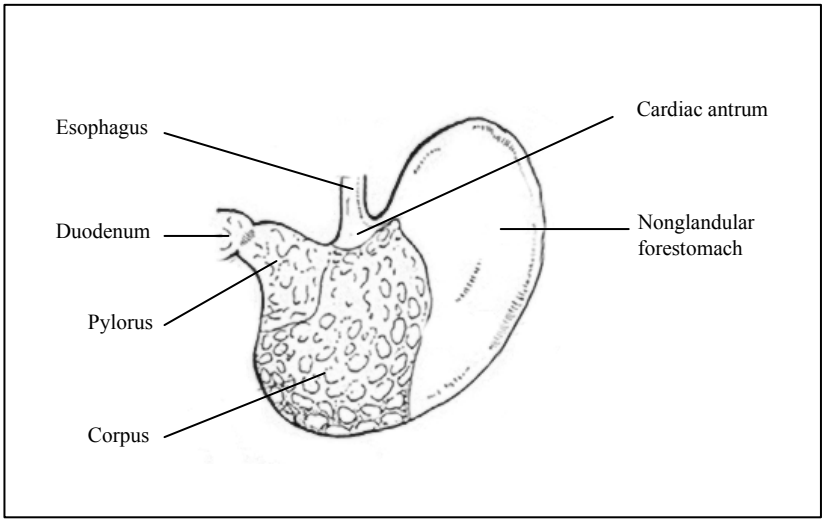

Fig. 4. Drawing of anatomical regions of the mouse stomach from the ventral aspect.

The wall of the mouse stomach is made up of four histological layers: mucosa, submucosa, muscularis and most outer serosa. The mucosa consists of the epithelium and the lamina propria, composed of connective tissue with scattered mesenchyme cells. Under the mucosa is a thin layer of smooth muscle, the muscularis mucosae (Kaufman, 1992; Ramalho-Santos *et al.*, 2000).

The marked difference to the human stomach, grossly visible in the mouse stomach, is the division into right thick-walled and left thin-walled portion (most of the body). These two functionally distinct regions are separated by the cardiac antrum, where the esophagus enters into the stomach (Fig. 4). The proximal, thick-walled forestomach (non-glandular) is lined by squamous keratinized stratified epithelium similar to and continuous with the esophagus. The thin-walled left side (glandular) has folded glandular epithelium of columnar cells (Kaufman, 1992; Karam, 1998).

1.3.3. Organization of the Gastric Epithelium in the Mouse

One of the fundamental processes during embryonic development of the stomach is the continuous self-renewal of the epithelium from stem cells, which persists during the post-natal and adult animal life (Leblond *et al.*, 1959). Despite the marked differences in the rates of differentiation, migration and life span, gastric and intestinal epithelia have the capacity of establishment and maintenance of a precise equilibrium among all the cellular components. Because of this feature stomach and intestinal epithelia have been used as a suitable model to address many basic questions of cell biology (Gordon and Hermiston, 1994). The gastric mucosa contains several differentiated epithelial cell types specialized in the secretion of acid, digestive enzymes, mucus, and hormones (Karam and Leblond, 1992). Differentiated cell lineages fulfilling this functions are organized together with their precursors into tubular invaginations that represent the functional and structural unit of the glandular epithelium called pit-glands (Fig. 5). In these units, along the pit-gland axis, occurs the entire process of proliferation, commitment, migration-associated differentiation, and finally the cellular death. Structure and function of the pit-glands depends on their localization in the three glandular regions of the stomach (Rubin *et al*., 1994; Fig. 5A):

1) Pure mucous zone, where epithelial cells of the pyloric antrum form long pits, continuous with short mucus-producing gland tubules.

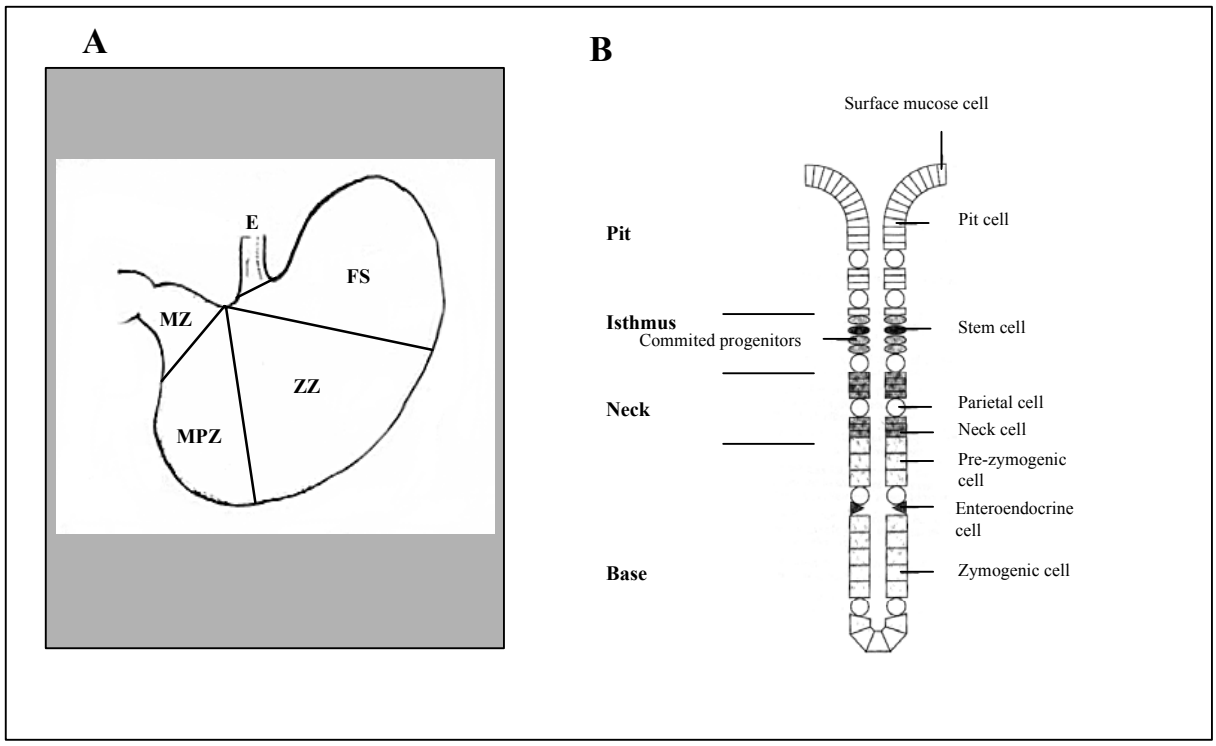

Fig. 5. Organisation of mouse gastric epithelium and lineage relationships of its component cell population. **(A)** Drawing of cross section along the cephalocaudal axis is showing oesophagus (E), forestomach (FS), and regional division of glandular epithelium into a zymogenic zone (ZZ), a mucoparietal zone (MPZ), and a pure mucous zone (MZ). **(B)** Schematic view of a gastric unit in the zymogenic zone of the glandular epithelium of an adult mouse (Gordon and Hermiston, 1994; modified).

The largest central corpus region of the stomach is subdivided into 2) proximal zymogenic zone and 3) middle mucoparietal zone. The epithelial cells from the corpus region form short pits continuous with long tubular glands (Gordon and Hermiston 1994; Karam, 1998; Karam, 1999). Histologically the transitions between the pyloric antrum and the corpus of the stomach is characterized by gradual and marked disappearance of pepsinogen and intrinsic factor producing cells (chief cells) and acid producing cells (parietal cells) towards the pylorus.

1.3.4. Main Cell Types along the Mouse Gastric Pit-Gland Axis

The cell lineage relationship in the gastric unit can be demonstrated on the zymogenic pit-gland of the corpus region of the stomach (Fig; 5B).

In the mouse the zymogenic pit-gland unit is lined by a monolayer of an average 200 epithelial cells (Karam and Leblond, 1992) mostly representing five principal lineages: mucus producing pit cells, neck cells, acid producing parietal cells, zymogenic cells synthesising pepsinogen and intrinsic factor, caveolated cells with unknown function, and

enteroendocrine cells, which elaborate a variety of neuropeptides. The apical part of the pit-gland unit, known as the pit region, forms the entrance into the unit from the luminal surface. The pit region is populated with ~40 mucus-secreting pit cells, characterized by dense mucus granules organized in ectoplasm (Karam and Leblond, 1993b). The pit is followed by the centrally located isthmus that contains one or more multipotent stem cells and its committed, undifferentiated descendants (Karam and Leblond 1993a). The neck region, just below the isthmus, comprises of the mucus/pepsinogen-secreting neck cells. There are about 13 neck cells per gastric unit producing prosecretory vesicles that are scattered in the cytoplasm. The tubular gland is terminated with a base region. This segment is inhabited by ~70 pepsinogen-secreting zymogenic cells (chief cells) (Karam and Leblond, 1993c). The production of pepsinogen increases in zymogenic cells in comparison to the neck cells together with the size of pepsinogenic granules and size of the amount of rough endoplasmatic reticulum. The acid-secreting parietal cells (oxyntic cells) are scattered along all the regions of the unit. All together there are about 26 parietal cells per unit and they are characterized by long numerous microvilli lining canalicular/apical membranes and large mitochondria (Karam and Leblond, 1993c).

During development of gastric epithelium the first typical parietal, pit and neck cells appear already at embryonic day 18 (E18). Fully differentiated zymogenic cells are detectable as late as on postnatal day 21 (P21) when the development of gastric epithelium is considered to be completed (Gordon and Hermiston, 1994; Rubin *et al.*, 1994).

1.3.5. All Stomach Epithelial Cell Lineages Originate from Multipotent Stem Cells

The stem cells of the gut are anchored in a specific location. The proliferative zone of gastric units is called isthmus (Fig. 5B). The stem cells located in the isthmus exhibit embryonic cell like features. They have high capacity of proliferation to ensure self-renewal and to produce uncommitted and/or committed precursor cells. The uncommitted precursors show dual characteristics of the stomach cell lineages and eventually turn into committed precursor cells (Karam and Leblond, 1993a). Committed precursors already have characteristics of one cell lineage lining the gastric pit-gland by expressing of specific genes. The actively functional cells are the differentiated mature cells.

The cell differentiation occurs during upward and downward migration from the isthmus region. Among the principal epithelial cell lineages, only the parietal cells differentiate within one day in the proliferative isthmus zone itself. Already mature parietal

cells undergo bipolar migration towards the pit region and down to the neck and base region. Mucus-producing pit cells differentiate through pre-pit cell precursors during upward migration to the surface where they eventually undergo apoptotic or necrotic death. Members of the zymogenic cell lineage migrate downward and differentiate first through the stage of pre-neck cells into neck cells in the neck region. There they function as mucus/pepsinogen-secreting neck cells. When the neck cells reach the base they become pre-zymogenic cells. They produce larger granules that are entirely filled by pepsinogen. Zymogenic cells subsequently acquire the ability to produce intrinsic factor as they complete their translocation to the lower portion of the base. At this stage, the cells are functional zymogenic cells (Karam and Leblond, 1992; 1993c; Karam, 1999). The relation among the principal cell lineages is depicted in figure 5B.

1.3.6. Control of Proliferation and Differentiation of Stomach Epithelium

The pathways that regulate the proliferation and differentiation processes in gastric epithelium still remain unclear.

In studies using gastric embryonal (E14) isografts it was shown that both, proliferation and differentiation, together with axial patterning of stomach glandular epithelium, are independent of the luminal content exposure (Rubin *et al.*, 1994).

The signals that epithelial cells receive to start their proliferation and to execute their differentiation programs are sent from the mesenchyme via direct cell-cell contact or by diffusion of soluble factors and from the extracellular matrix (Birchmeier and Birchmeier; 1993; Gumbiner, 1996). It seems also possible that epithelial cells themselves, as it is known for the parietal cells, could be a source of factors important for differentiation (Li *et al.*, 1996; Canfield *et al.*, 1996).

In expression hierarchy, transcription factors influence local expression of cell signaling molecules. That leads to modulation of cell adhesion. To date several transcription factors, growth factors and their receptors, and adhesion molecules have been identified that are expressed in the gastric epithelium or mesenchyme. However, dissection of the signaling pathways and determination which of the potential mediators actually regulate differentiation in the stomach, and GI tract in general, was still not reported (Lengyel and Lui, 1998). The obstacle is obvious. First of all the dynamic complexity of stomach epithelium is irreproducible *in vitro*. The gene targeting, one of the most powerful methods for studying the physiological roles of the genes, as well has its harshness. The weakness of

this method is the time point when the gastric epithelium starts its differentiation programs. Since this occurs in the late stage of embryonic development, the targeted animals die too early to determine the role of the mutated genes (HNF3β, SF/HGF, c-Met, E-cadherin, β-catenin, γ-catenin, Smad4/ Dpc4, Ang and Rossant, 1994; Schmidt *et al.*, 1995; Bladt *et al.*, 1995; Uehara *et al.*, 1995; Larue *et al.*, 1994; Haegel *et al.*, 1995; Ruiz *et al.*, 1996; Yang *et al.*, 1998). For other targeted genes the stomach mucosa develops normally (TGFα, TGFβ1, IGF1 and IGF1 receptor, EGFR, HNF3γ; Shull *et al.*, 1992; Liu *et al.*, 1993; Luetteke *et al.*, 1993; Mann *et al.*, 1993; Sibilia and Wagner, 1995; Kaestner *et al.*, 1998).

1.3.7. Cell Adhesion - the Key to Morphogenesis of the Epithelium and Maintenance of the Epithelial Architecture

As the stomach epithelial progenitors migrate along the pit-gland axis they differentiate. Signaling that leads towards lineage precursors and finally fully functional cells is not selfautonomous.

The morphogenesis of the epithelium is a reflection of an interplay between various adhesion receptors, the cytoskeleton, and the coordination of transduction pathways. The assembly into a three-dimensional tissue structure is a result of adhesion mechanisms. The physical aspect of the adhesion, in the meaning of maintenance of the epithelial structure, is coupled with the capacity of adhesion molecules to respond to cell-cell or extracellular matrix (ECM)-cell signaling events and to transduce signals into the cells.

The adhesion molecules are usually glycoproteins localized on the extracellular surface. To keep the architecture of the epithelium, four kinds of stable adhesion elements are important: adherens junctions; desmosomal junctions; occluding junctions; and attachment to the extracellular matrix (ECM).

Adherens junctions are one the most important adhesive elements that play a pivotal role in the establishment and maintenance of the unique tissue architecture. Their ubiquitous presence is required for tight association among epithelial cells and is mediated mostly by E-cadherin, as well as by recently identified afadin (Ikeda *et al.*, 1999). E-cadherin belongs to the classic cadherins and as a single transmembrane domain protein is involved in calcium dependent homophilic cell-cell recognition and adhesion during the development (Takeichi, 1991; Hynes, 1992a; Gumbiner, 1996).

Loss of E-cadherin was associated with cancer progression, thus it is thought E-cadherin functions as an important tumor suppressor (Birchmeier and Behrens, 1994).

E-cadherin is linked to the actin cytoskeleton by the cytoplasmic catenins (Cowin and Burke, 1996). ∝-Catenin links E-cadherin to the actin cytoskeleton through β- or γ-catenin. Although the cell-cell adhesion mediated by E-cadherin-catenin complex is strong, this complex is permissive to cell rearrangement and tissue morphogenesis in response to growth factors. E-cadherin sensitively answers to local signaling, but it as well participates in long-range developmental patterning processes in the embryo through the cytoplasmic β-catenin, an essential component of the wnt signaling pathway (Miller and Moon, 1996).

The E-cadherin-catenin complex adhesion was found to be regulated by several direct and indirect mechanisms (Gumbiner, 2000). Apart from the wnt pathway through modulation of β-catenin levels, the small GTPases, Rac, Rho and Cdc42 can regulate actin-membrane interactions. IQGAP1, an effector of Cdc42 and Rac1, has a direct but not essential effect on the cadherin complex (Kuroda *et al.*, 1998). IQGAP1 competes with α-catenin to bind the E-cadherin-β-catenin complex and thus renders the cells non-adhesive.

β-Catenin and γ-catenin are both present in adherens junctions. γ-Catenin is also present in *desmosomal junctions* where it interacts with desmogleins and desmocollins (Ben Ze'ev and Geiger, 1998). Desmosomes are as well important elements in epithelia. Desmogleins and desmocollins are the adhesion receptors of desmosomes. They are linked to the microfilament network by cytoplasmatic proteins, like plakoglobin. The role of desmosomal junctions is the maintenance of tissue integrity.

Occluding (tight) junctions form the selectively permeable barrier separating two functionally and biochemically distinct regions. Tight junctions are typical for single-layered epithelium and for the endothelium at their apical and basolateral surface. The level of permeability of tight junctions varies in dependence on tissue specificity and ranges from whole cells to ions and protons (Gumbiner, 1996).

The attachment of cells to ECM and to its more distinct sheath - a basement membrane - crucially contributes to tissue integrity, cell polarization and branching morphogenesis of the epithelium (Hynes, 1992b; Gumbiner, 1996; Drubin and Nelson, 1996). Different cell adhesion receptors are endowed with interaction to ECM, including a family of cell surface proteoglycans - syndecans. The most conspicuous adhesion molecules mediating cell adhesion to ECM are integrins representing a family of transmembrane, heterodimeric proteins. Similar to cadherins, integrin mediated adhesions induce the localized assembly of specialized cytoskeletal and signaling networks at the contacting cell surface. Integrins are linked through α-actinin and talin to actin filaments and actin-associated proteins, including

paxillin, zyxin, vinculin and a number of protein kinases (Gumbiner, 1996). ECM are thought to contain highly regionalized signals that are needed to control the patterns of morphogenesis.

Cell adhesion is an important factor in the assembly of the dynamic, three-dimensional order of epithelial cells. Adhesion molecules are sensitive mediators of a variety of signaling pathways leading towards the morphogenesis of developing tissues. Thus the coupling of both - the physical adhesion and signal processing - ensure the fully functional status of the epithelium.