Multifunctionality of proteins is among mechanisms accounting for the complexity of interactome networks in higher eukaryotes. During oncogenesis and other pathologic conditions many proteins perform additional functions without changes in three dimensional structures. One family of these moonlighting proteins is represented by enzymes and cofactors of aminoacylation reactions, by means of which tRNAs are attached to their cognate amino acids. Tyrosyl-tRNA synthetase (TyrRS), tryptophanyl-tRNA synthetases (TrpRS) and auxiliary factor of mammalian multi-aminocyl-tRNA synthetases, p43 (precursor of endothelial monocyte activating polypeptide II — EMAP II) upon their release in intracellular environment become proinflammatory cytokines with multiple activities during apoptosis, angiogenesis and inflammation. In addition, these proteins play important role in cancer progression, modulating tumor angiogenesis and its escape from surveillance by immune system. 

Key Words: moonlighting proteins, endothelial-monocyte activating polypeptide II, tyrosyl-tRNA synthetase, tryptophanyl-tRNA synthetase, apoptosis, tumor angiogenesis.

Although multicellular organisms have very complex organization, the size of their genomes is relatively small in comparison with unicellular species [40]. One explanation for such discrepancy is the availability of mechanisms that allow synthesis of several proteins from one gene (for example, through alternative splicing). But even these mechanisms could not explain functional variability of proteomes in higher eukaryotes. Recently discovered concept of multifunctional proteins is special characteristic of mammalian cells that can significantly expand a number of encoded functions. Three dimensional protein structure could contain motifs that allow it to perform different functions with very insignificant structural changes. It is becoming clear that functions of protein could vary greatly depending on its intracellular localization and on general physiological state of the cell [10]. Obviously, both alternative splicing and post-translational mechanisms responsible for protein variability are not exclusive and functional variability of most vertebrate proteins is regulated by both mechanisms [12].

Malignant transformation is an example of drastic changes in cellular state and it is characterized by pathological changes in structure and function of many onco-genes and suppressor genes encoded proteins ultimately leading to malfunctioning of many biochemical and signaling pathways. Apart from oncogene—encoded proteins, many other housekeeping proteins of cytoskeleton, cellular adhesion and different biochemical and gene expression pathways are also involved in transformation process. One of the families of multifunctional proteins is represented by some aminocycl-tRNA synthetases and cofactors of aminoacylation reaction. The major housekeeping function of aminocycl-tRNA synthetases is attachment of amino acids to their cognate tRNAs during translation. In mammals additional cofactors — proteins p18, p38 and p43 form the core of aminocycl-tRNA synthetase complex, which assist the aminoacylation. However, two synthetases in mammals, tyrosyl—tRNA synthetase (TyrRS) and tryptophanyl—tRNA synthetase (TrpRS) and p43 cofactor (Figure), upon release in intracellular space gain additional activities and began to act as cytokines, regulating migration, division, differentiation and apoptosis of various cells of immune system and endothelial cells, and participating in malignant transformation. Although cytokine functions of these proteins have been investigated for more than 12 years, the mechanisms of their multifunctionality have yet to be discovered. In this article we will briefly review present state of research on cytokine activities of these proteins and their implication in carcinogenesis.

**EMAP II/p43.** Cytokine activity of p43 has been discovered in 1992 when it was purified from methylicholanticren A induced (Meth A) mouse fibrosarcoma and named endothelial and monocyte activating polypeptide II (EMAP II) [13]. Endothelial cells of mouse fibrosarcoma have very high sensitivity to many cytokines, and consequently can be used as a good model for studying angiogenesis factors. EMAP II, purified from these tumors, appeared to be a small protein with molecular weight of 22 kDa, but cDNA of the gene cloned later suggested the existence of 34 kDa EMAP II precursor (proEMAP II). Purified recombinant EMAP II elevated expression of P and E—selectins on the surface of endothelial cells, raised level of intracellular calcium and induced tissue factor. Also EMAP II activated neutrophils (chemotaxis, release of intracellular calcium and peroxidase) and monocytes (stimulation of chemotaxis, induction of tumor necrosis factor (TNF) and tissue factor) [14]. Local intratumoral injection of EMAP II led to bleeding and significant reduction in tumor size. Tumors, treated with EMAP II, also demonstrated higher sensitivity to TNF—
duced apoptosis. Despite so many cytokine activities, protein sequence of proEMAP has no signaling peptide for secretion into endoplasmic reticulum or amino acid residues for attachment of PI3 anchors, which would explain how EMAP II may be transported to plasma membrane.

Studying the mechanisms of EMAP II processing and transport to intracellular space, Knies et al. [20] have proposed the model for EMAP II proteolysis that utilize involvement of apoptosis pathways. Immunocytochemical analysis and mRNA in situ hybridization of early mouse embryos showed that EMAP II is transported to the regions that undergo a lot of restructuring and have many apoptotic cells [20]. In addition, western blotting proved that apoptosis, but not necrosis, induced intracellular release of 22 kDa form of EMAP II and some other products of proteolysis. Authors have used several inhibitors of caspases to suggest that EMAP II processing is a result of peptide bond cleavage after asparagine residue (Asp 144) [39]. Thus, according to this model, EMAP II provides “clearance signal” during apoptosis and promotes migration of macrophages to the regions with many apoptotic cells [20]. In addition, western blotting proved that apoptosis, but not necrosis, induced intracellular release of 22 kDa form of EMAP II and some other products of proteolysis. Authors have used several inhibitors of caspases to suggest that EMAP II processing is a result of peptide bond cleavage after asparagine residue (Asp 144) [39]. Thus, according to this model, EMAP II provides “clearance signal” during apoptosis and promotes migration of macrophages to the regions with many apoptotic cells [20].

Figure. Domain organization of tyrosyl–tRNA synthetase, tryptophanyl–tRNA synthetase and endothelial monocyte activating polypeptide II

<table>
<thead>
<tr>
<th>Tyrosyl–tRNA synthetase</th>
<th>Aminoacylation cofactor p43</th>
<th>Tryptophanyl–tRNA synthetase</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Catalytic module</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rossmann fold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-helical domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>COOH-terminal module</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OB-fold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-subdomain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>528 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asp144</td>
<td></td>
<td></td>
</tr>
<tr>
<td>312 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Catalytic module</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rossmann fold</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-helical domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>471 aa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It has been demonstrated that EMAP II could trigger apoptosis of endothelial cells in vitro [3, 38]. Interestingly, proapoptotic activity of EMAP II is observed only with high EMAP II concentrations (more than 100 µg/ml). Transgenic tumor cells that synthesized higher amounts of EMAP II were characterized by slow growth and low rate of metastases formation. Unfortunately it is not clear how EMAP II transmits signal intracellularly to induce the apoptosis and which receptor/receptors it uses.

Because in many cytokine assays EMAP II activity increases with incorporation of TNF, it may be suggested that EMAP II acts through low-affinity coreceptor to TNF receptor 1 (TNF–R1) [7, 45]. One of the early effects of EMAP II on endothelial cells is the induction of elevated expression of TNF–R1 [4]. In procoagulation assay of endothelial cells, TNF administered along with EMAP II caused 20 folds increase of its activity [1]. In our experiments EMAP II and TNF (at the concentration of 10 pM) stimulated the release of tissue factor by neutrophils at the concentration of 65 pg/10⁶ cells, but upon their separate addition the release of tissue factor was much lower — 2 pg/10⁶ cells for EMAP II (10 pM) and 9 pg/10⁶ cells for TNF (10 pM). EMAP II and TNF also may activate transcription of each other in endothelial cells and macrophages. These data indicate close relationship between EMAP II and TNF functions. For example, during oncogenesis TNF could stimulate apoptosis of endothelial cells by induction of EMAP II
transcription and promoting EMAP II release in tumor cells, thus accounting for slower tumor growth.

What particular EMAP II structural motifs can attribute to its cytokine activities? Early experiments with site-directed mutagenesis showed that hexapeptide \(^{158}\text{RVGKII}^{164}\) could play some role in cytokine functions of EMAP II [15]. However, as crystallographic analysis had shown, residues 158, 159, 160 and 161 of the hexapeptide are located in hydrophobic core and participate in formation of intradomain interface. Because release of this hexapeptide would require cardinal changes and partial denaturation of protein structure, currently its role in cytokine activities is under doubt [35]. It is most likely that amino acid residues required for cytokine activities span large region of EMAP II three-dimensional structure. This assumption is supported by the fact that both COOH-terminal 22 kDa module (EMAP II) and NH\(_2\)-terminal module of p43 have similar cytokine activities (though slightly lower than those of the full-length protein) [32].

At present time the protein 3D structure is known for EMAP II in different crystallographic form [16, 35]. EMAP II forms compact globule composed from two domains of 100 and 66 amino acids tightly associated through interdomain interface. The larger domain at NH\(_2\)-terminus (aa 148–252 in p43) forms open \(\beta\)-barrel–distinguishing feature of OB–folds (oligonucleotide binding) presented in many RNA binding proteins. On the contrary, smaller domain (A subdomain, aa 253–312 in p43) has no homology to other mammalian proteins. S. Quevillon et al. [34] proposed the hypothesis of evolutionary origin of A–subdomain where it evolved from oligomeric protein similar to bacterial Trbp (tRNA–binding) that contain OB–fold. According to this model, one of OB–folds in Trbp evolved into A–subdomain through a number of deletions of \(\beta\)–strands in a secondary structure, and later combined with the second OB–fold. Such assumptions are also supported by relatively high structural similarities between OB–fold and A–subdomain.

As we have mentioned before, precursor of EMAP II p43 has similar to EMAP II cytokine activity [21]. Furthermore, experiments have shown that p43 is constantly secreted by tumor cells, albeit in low quantities. p43 secretion was also increased after induction of apoptosis in 293 cell line starting from 30 min of treatment, but EMAP II appeared only at 12 h. Microarray analysis of p43 secreted by 293 cell line during the period of apoptosis revealed induction of TNF (30 fold), macrophage inflammatory proteins, ICAM–1 adhesion molecules and several other cytokines and chemokines involved in inflammation [20]. Because EMAP II is not observed at early stages of apoptosis, it is possible that p43 could be the major protein form in angiogenesis and other processes, revealing its multifunctional nature. This hypothesis is in agreement with first reports on EMAP II purification, because EMAP I detected alongside with EMAP II could have been p43 (EMAP III turned out to be endothelial growth factor).

S. Kim et al. [32] have performed experiments similar to those of A. Berger [3] that involved treatment of endothelial cells with EMAP II, but this time full–length p43 was used. Results demonstrated that while at high doses (more than 100 nM) p43 induced apoptosis of endothelial cells, at the low doses (around 1 nM) it promoted their migration and acted like proangiogenic protein. High reliability of results stems for the fact that similar data were obtained in three independent angiogenesis assays. Further experiments have demonstrated that different concentrations of p43 activated different intracellular pathways. Low doses of p43 activated EPK kinase that control expression of matrix metalloproteinase 9. The later hydrolised collagen and thus promoted endothelial cell migration. On the other hand, higher doses of p43 stimulated proapoptotic kinase JNK and this can be one of the mechanisms for p43–induced apoptosis of endothelial cells (complete inhibition of JNK reduced, but not completely abolished proapoptotic activity of p43) [33].

What role EMAP II/p43 plays in tumor angiogenesis is still unclear. Tumor endothelial cells differ significantly from normal endothelium — former are more heterogenic, less organized and differentiated as a result of aberrant cytokine stimuli from cancer cells [5]. It is possible that cancer cells secret EMAP II/p43 in the way that promotes migration of endothelial cells. Interestingly, benign tumors, characterized by large number of hypoxic cells, synthesize higher concentration of p43 [25]. Apart from full–length EMAP II, hypoxic cells secret EMAP II (22 kDa form) and several intermediate forms (22 kDa–27 kDa). Here elevated EMAP II expression may be considered as a defensive mechanism of mammals against malignant transformation. Furthermore, hypoxia–induced EMAP II secretion is caspase independent and consequently may represent another pathway of EMAP II proteolysis.

Constant EMAP II/p43 secretion by endothelial cells indicates that this protein had additional functions useful to tumor cells not restricted by its concentration–dependent influence on endothelial cells. Murray et al. [30] discovered a membrane–bound form of EMAP II/p43 on cancer cells that may induce apoptosis of lymphocytes and helps the tumor to escape surveillance by immune system. At the same time, for induction of apoptosis in lymphocytes it is necessary to use doses as high as 40 nM, while concentration of EMAP II in cultural medium does not exceed 0.5 nM. The reasonable assumption is that natural EMAP II undergoes some post–translational modifications or and co–act with other factors for apoptosis induction. EMAP II has also been detected in tissues after injury, but its physiological role in inflammation is still undetermined [26–28].

The major question regarding EMAP II/p43 multifunctionality pertains to regulation of its cytokine activities on translational and posttranslational level. Transcription of human p43 is activated by treatment with cytosolic cytokines like IFN–γ and TNF, which ultimately resulted in the raise of intracellular EMAP II/p43 level. Such uncontrollable increase could influence stability of multisynthetase complex by changing its stoichiometry. Possibly, the post–translational processing could be performed by different pathways, one of which could safely export EMAP II/p43 outside the cell; or selective mRNA degradation might regulate EMAP II/p43 abundance in cytoplasm. Both EST and SAGE data indicated the presence of two alternatively polyadenylated transcripts of EMAP II/p43 mRNA. We analy—
sed affymetrix microarray data and found that two probe sets covering regions of alternative poly A sites gave rise to different EMAP II mRNAs with distinct expression levels in a number of cancer tissues [9]. Such drastic differences in abundance of alternative EMAP II/p43 transcripts could be explained by greater instability of longer 1764 bp EMAP II/p43 mRNA that have 11 oligonucleotide repeats UAUUUUAU in its 3’ untranslated region. This region is similar to polyU regions in TNF mRNA, which confer susceptibility to a rapid mRNA degradation. Thus, in this model production of 1764 bp EMAP II mRNA can lead to the rapid rise in the EMAP II level, following by its very quick degradation and without any deleterious effect on stoichiometry of mammalian multisynthetase complex.

As we can see from this brief review, p43 — housekeeping protein that participates in translation, may take part in the interactions of tumor and immune system. During vertebrate evolution both tumor and normal cells used EMAP II/p43 for their benefits and its different functions can be regulated on the level of cellular localization, protein concentration in intracellular space and cooperation with other cytokines. Depending on acceptor cell, cytokine effect of EMAP II/p43 may also vary. EMAP II/p43 is perspective molecule for potential clinical applications, but further investigations of its biology are required before this protein may be used for therapeutic purposes.

**TrpRS.** TrpRS was the first aminoacyl–tRNA synthetase for which cytokine activities have been proposed [18]. In contrast to p43 and TyrRS, TrpRS is regulated not only at the level of posttranslational proteolysis, but also at the levels of transcription initiation and alternative splicing. As early as in 1991, it was shown that gene expression of TrpRS is regulated by interferon–γ (IFN–γ) [6].

At present time, 6 alternative mRNAs, whose abundance is regulated by splicing, are known for TrpRS, and INF–γ is responsible for their production [24]. One of the major mRNAs encodes protein isoform (mini TrpRS) with reduced NH2-terminus where translation begins from the 3’ end of the gene that encodes this partial protein. TrpRS is cleaved by aminopeptidase and aminopeptidase that result in formation of two amino acids. As we can see from this brief review, p43 — housekeeping protein that participates in translation, may take part in the interactions of tumor and immune system.

**Aminoacyl–tRNA synthetases of higher eukaryotes have additional NH2- and COOH-terminal domains, that are absent in their bacterial homologues and do not have enzymatic functions. Research on cytokine activities of TrpRS has begun from the observation that aminoacid sequence of COOH–terminal domain of TyrRS (C–module) shares 53% sequence similarity with EMAP II [19, 22]. Homology at the structural level is even higher — root mean square deviation (RMSD) between OB–folds of TyrRS and EMAP II is 0.6 Å, which is equivalent to RMSDs between different crystallographic structures of the same protein. Similar to EMAP II, C–module of TyrRS stimulates the migration of monocytes and release of TNF, peroxidase and tissue factor [22]. In our assays on neutrophile migration and tissue factor secretion, C–module of TyrRS showed the lower activity than EMAP II/p43; it could be explained by the use of recombinant proteins (bovine TyrRS used for recombinant protein production shares 94.3% identity with human TyrRS).

Unexpected results gave cytokine assays for NH2–terminal catalytic fragment of TyrRS (mini TyrRS) [42]. It was discovered that mini TyrRS may be chemo tactic for neutrophils and may stimulate angiogenesis in a concentration–dependent manner (angiogenesis is induced at concentrations > 60 pM). ELR motif found in many CXC-chemokines is thought to account for these functions. It is located in the Rossman fold of catalytic domain and mutations of arginine in the motif abolished cytokine activity of mini TyrRS. As in the case with EMAP II/p43, receptor for mini TyrRS has not yet been found, although it has been shown that mini TyrRS can bind to the receptor B for interleukine–7β [41]. Also, TyrRS mRNA does not encode signaling peptide for transportation of protein to the endoplasmic reticulum.

According to the model [41], TyrRS is secreted into intracellular space during apoptosis, where it is cleaved by leukocyte elastase into NH2– and COOH–terminal fragments (full–length TyrRS, in contrary to EMAP II, has no cytokine activities). However, we have not enough data to exclude the possibility of intracellular TyrRS proteolysis. While ASTD motif, which is cleavable by caspase 7, joins NH2– and COOH (EMAP II) domains of p43 [2], C–module of TyrRS is attached to catalytic domain through PEST–like sequence. PEST sequences are rich in proline (P), serine (S), glutamic acid (E) and threonin (T) residues and they are natural substrates for cleavage by many intracellular proteinases [36]. Consequently, pathological processes that lead to release of proteinases could trigger proteolytic cleavage of TyrRS. For example, in our experiments TyrRS was easily cleaved by trypsin (Kmelyuk et al, unpublished observations).

Returning to the discussion on proangiogenic activities of TyrRS fragments, we should mention that although C–module have not yet been assayed for angiogenic activities, it is highly probable that it possesses them. First of all, C–module is highly similar to EMAP II that exhibits angiogenic activities. Our bioinformatics analysis of TyrRS upstream sequences identified a number of interferon–inducible elements, which implicates possible regulation of TyrRS by interferon family of cytokines [9]. As INF–γ inducible expression is characteristic for many proinflammatory and antiangiogenic proteins, the fact that TyrRS upstream sequences harbor interferon family of cytokines could have possible functional role in cells angiogenic response to pathological conditions. The fact that
NH₂- and COOH fragments of TyrRS have competently different angiogenic properties can be explained from the following perspective. Endothelial cells are characterized by high apoptosis rate and growth dynamics [8]. Consequently, release of two antagonistic in their activities proteins from tumor cells may be one of the factors responsible for high plasticity of endothelium. Because development of malignant phenotype is partially dependent on angiogenesis rate, balance between proangiogenic and angiostatic factors plays crucial role in the development of cancer. The shift in this balance may lead to malignant transformation of cells. Therefore, tumor cells could have independent of apoptosis mechanisms for TyrRS transportation into intracellular space.

The examples of proteins involved in tRNA aminoa- cyclation reaction illustrate the importance of protein multifunctionality in higher eukaryotes. Among other proteins we should mention the function of mammalian IL-1α as a transcription initiation factor [44], or the role of glucose-6-phosphate isomerase in migration of cancer cells [11, 37, 43]. One possible way for development of multifunctional proteins is a participation of housekeeping proteins in various processes in immune and nervous systems. Mammals differ from other vertebrates by more complex organization of these systems, which evolved through complexification of protein three-dimensional structure and formation of multidomain proteins. During oncogenesis, a number of proteins attain new noncanonical functions, thus promoting malignant transformation of cell.

REFERENCES