The Role of Tyrosine Phosphorylation in Regulation of Signal Transduction Pathways in Unicellular Eukaryotes

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Abstract

The review summarizes for the first time the current concepts of the role of tyrosine phosphorylation in regulation of signal transduction pathways in unicellular eukaryotes. Evolutionary concepts are developed about the origin of protein tyrosine kinases (PTK)-signaling.

Introduction

At the end of the last century, in mammalian cells a multicascade pathway was discovered to be responsible for transmission of proliferative signals into genome. The key role in the signal transmission through this pathway is played by processes of phosphorylation of protein tyrosine residues (Hunter, 1995; Marshall, 1995; Feig *et al*., 1996; Mustelin *et al*., 2002).

Ushiro and Cohen (1980) were the first to establish the important role of phosphorylation of tyrosine as a regulator of intracellular processes and to reveal changes of tyrosine kinase activity of proteins in mammalian cells in response to action of growth factors. Subsequently, the change of the protein tyrosine kinase activity was shown to underlie the Ras-MAPK signaling pathway regulated by Mitogen-Activated Protein (MAP) kinases (Hunter and Cooper, 1985).

Phosphorylation of tyrosine residues in proteins is catalyzed by retrovirus protein tyrosine kinases (PTKs), their cellular homologues (Bishop, 1983) or by tyrosine kinases associated with growth factor receptors (Yarden and Ullrich, 1988; Broome and Hunter, 1996). These proteins were studied in detail and classified in vertebrate animals (Hanks and Hunter, 1995).

The classical scheme of transmission of the proliferative signals through the pathway mediated by growth factors (Ras-MAPK pathway) includes: (1) association of growth factor with receptor, (2) dimerization of receptor and autophosphorylation of receptor tyrosine kinase (RTK), (3) module coupling of RTK with adaptor SH2–domain proteins; activation of Ras, (4) phosphorylation and activation of MAP kinases, (5) transmission of signal into genome.

At the end of the last century, another pathway of transmission of proliferative signals into genome, with participation of growth factors and tyrosine kinases, was discovered (Shuai *et al*., 1994). This is the monocascade STAT pathway activated by receptors of growth factors and cytokines. The essence of this transmission consists in direct activation by tyrosine kinases of the STAT (signal transducer and activator of transcription) proteins located in the cytoplasm. This transmission is also provided by the SH2–domain contacts responsible for coupling of phosphotyrosine-containing proteins (Darnell, 1997).

The structural-functional organization of Ras-MAPK and STAT signaling pathways in vertebrate cells has been intensively studied for more than 20 years. By the present time an extensive experimental material has been accumulated, which allows judging about the events in cells of the higher eukaryotes at each stage of transmission of proliferative signal. Much less is known about mechanisms of transmission of growth signals in cells of primitive eukaryotes. There are practically no data in the literature about the existence of their STAT and Ras-MAPK pathways of the signal transmission; meanwhile, it is obvious that RTKs exist not only in mammals, but in multicellular invertebrates, including the most primitive — sponges and coelenterates (Schartl, Barnekow, 1982; Schartl *et al*., 1989; Kruse *et al*., 1997*)*.

Our analysis of experimental and literature data has shown that tyrosine phosphorylation also takes place in cells of unicellular eukaryotes. The presence in eukaryotic microorganisms of tyrosine kinases and proteins phosphorylated at tyrosine in processes of growth, cellular differentiation, and adaptive response indicates a more ancient origin of mechanisms of PTK-signaling than this was believed earlier.

This review focuses on the role of mammalian growth factors, tyrosine kinases, and phosphotyrosine-containing proteins in signaling in unicellular eukaryotes.

Effects of mammalian growth factors on unicellular organisms

Due to the accepted concept that PTK-signaling takes place only in cells of the higher eukaryotes, many aspects of the problem of evolutionary origin of this signaling are kept in the shade. Thus, for instance, nothing, in fact, is known about evolution of mechanisms of activation of the signal pathways dependent on tyrosine phosphorylation.

In this connection, it seems important to consider pathways of signal transmission by tyrosine phosphorylation in unicellular eukaryotes, whose cells have no typical growth receptors and RTKs.

It would have been logical to suggest that unicellular eukaryotes deprived of typical receptors of mammalian growth factors could not and should not respond to signals initiated by mammalian growth factors. However, in reality, this is not so. Studies performed for the last few years have shown that the mammalian growth factors (TGFα, EGF, FGF, HGF, etc.) can initiate proliferative effect not only in mammalian cells, but also in unicellular animals. The stimulating effect of mammalian growth factors on proliferative growth of eukaryotic microorganisms was revealed for the first time in the parasitic protozoa *Giardia lamblia* (Lujan *et al*, 1994). The mitogenic effect on

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growth of *G. lamblia* was produced by insulin-like factors IGF-I and IGF-II (Lujan *et al*, 1994). They were shown to accelerate growth and L-cysteine incorporation in *G. lamblia* trophozoites cultivated axenically in a serum-free medium. The insulin-like factors act through the parasite membrane glycoprotein that has immunological characteristics similar to those of the mammalian IGF-I. In reaction of immunoprecipitation of lysate of *G. lamblia* trophozoites incubated with human recombinant IGF-II, with monoclonal antibody αIR3, proteins of a molecular mass of 70 kDa were found; one of them, as shown by further studies using polyclonal antibody to the IGF-I receptor, corresponded to the IGF-I receptor α-chain. Protein of a molecular mass of 95 kDa that corresponded to the receptor IGF-I β-chain was discovered using polyclonal antibody to phosphotyrosine. Based on the obtained data, the authors suggested the existence in the membrane glycoproteins of *G. lamblia* trophozoites of internal tyrosine kinase activity and the capability for autophosphorylation.

The promoter effect of IGF-I on cell growth was also revealed in cells of the parasitic protozoa *Leishmania mexicana* (Gomes *et al*., 1998; 2001). The authors explain the stimulating IGF-I effect on leishmanian cells by a similarity of structure in receptors of leishmanian and mammalian cells. The receptor for IGF-I on *L. mexicana* promastigotes is a monomeric glycoprotein of a molecular mass of 65 kDa and is antigenically related to the α-chain of the human type 1 IGF-I receptor. Upon IGF-I stimulation the receptor undergoes autophosphorylation at tyrosine residues with activation of its signaling pathway. Activation of the IGF-I receptor also leads to phosphorylation of a 185-kDa molecule that is homologous to the substrate of the insulin receptor present in human cells, the insulin receptor substrate 1 (IRS-1) (Gomes *et al*., 2001).

By the example of free-living ciliates *Tetrahymena pyriformis* and *Tetrahymena thermophila*, the vertebrate insulin also was shown to be capable of changing the cell metabolism level and the growth rate of the protozoa (Christopher and Sundermann, 1996; Shemarova *et al*., 2002). In experiments on a combined use of epidermal growth factor (EGF) and insulin, it was found that the mitogenic effect on tetrahymena was evoked by progression of cells in G_1-phase under the action of growth factors and hence by an earlier entry into S-phase of the first cell cycle. Insulin repressed division of the cells entering the generative cycle, as the cells were delayed at the late S-phase and G_2-phase of the cycle; under these circumstances one part of the population was lost and another part overcame the cell block successfully and started dividing by 4 hr of cultivation (Shemarova *et al*., 2002).

The stimulating effect on growth of the parasitic protozoa *Trypanosoma musculi* was also produced by mammalian fibroblast growth factor (FGF). It was established that in the fibroblast-free serum medium, the *T. musculi* cells cultivated *in vitro* turned out to be unable to normally grow; they lost the capability for division and infectivity. These properties were retained only in trypanosomes growing in contact with FGF-producing fibroblasts. In immunocytochemical studies the parasites were found to contain the FGF-activated cytoskeleton and membrane-associated proteins that, in the authors' opinion (Gugssa *et al*., 2000), became involved in control of growth and reproduction of *T. musculi*.

Platelet growth factor (PDGF), whose receptors in mammals also have their own tyrosine kinase activity, stimulated macromolecular synthesis in the ciliate *Tetrahymena pyriformis* (Andersen *et al*., 1984). Measurements of changes in the labeled uridine specific activity in the UTP pool after PDGF treatment showed an initial increase lasting for about 40 min. Thereafter the RNA synthesis rate gradually decreased and became the same as in intact cells after 2 hr of incubation. PDGF also stimulated the labeled uridine incorporation in *T. pyriformis*. The increase begins shortly after addition of this growth factor and levels off 2–3 h later. The data obtained by these authors (Andersen *et al*., 1984) rule out a possibility of induction of changes in the nucleotide pool of precursors both of RNA and of DNA by PDGF in *T. pyriformis*. The authors suggested *T. pyriformis* to contain receptors on its surface for the growth factor.

Colony-stimulating growth factor (CSF) from granulocytes stimulated proliferative response and inhibited the heat-shock-induced apoptosis in *Leishmania mexicana amazonensis* (Charlab *et al*., 1990; Barcinski *et al*., 1992; Welburn *et al*., 1996). Some mammalian cytokines (IL-3, IL-6, TNF, and IFN-γ) are reported to induce in protozoan parasites a cellular reaction aimed at maintaining their viability (Olsson *et al*, 1992; Barcinski, Moreira, 1994; Csaba *et al*., 1995, Salotra *et al*., 1995; Bakhiet *et al*., 1996).

Several works have demonstrated stimulation of proliferation of protozoa by EGF (Hide *et al*., 1989; Sternberg, McGuigan, 1994; Selivanova *et al*., 2002; Shemarova *et al*., 2004). It was established that EGF produced a marked proliferative response *in vivo* in trypomastigotes of the *Trypanosoma brucei* strain Tc221 (Sternberg and McGuigan, 1994). A significant increase of the growth rate of the trypomastigotes was observed using both human recombinant EGF and the mouse salivary gland EGF at concentrations of 20 and 200 nM, respectively. The doubling time of the cell population for the first 24 hr of exponential growth for the trypanosomes incubated with EGF amounted to 5.9 hr, whereas for intact trypanosomes, to 6.7 hr. Similar results were obtained in experiments evaluating the EGF proliferative effect on *T. brucei* strain AnTaT1,1: the doubling time of experimental cultures was 5.6 hr *versus* 7.1 hr in intact cells. When trypanosomes were cultivated with addition of serum-free medium longer than for 24 hr, proliferation both of experimental and of control cultures gradually ceased. It was suggested that the proliferative EGF effect on trypanosomes was due to activation of the trypanosome EGFR-like membrane protein whose structure has a partial similarity with the extracellular domain of the human EGF receptor (EGFR) (Hide *et al*., 1989). Recently, new evidences have appeared in favor of structural similarity of individual fragments of the human EGFR extracellular domain and of EGFR-like protein of *Trypanosoma cruzi* amastigote forms (Ghansah *et al*., 2002). Scatchard analysis showed a single class of receptors with a Kd of 0.8 nM and numbering 3.1 x 10(3) per amastigote. Results from internalization experiments

provided evidence for receptor-mediated endocytosis of EGF. Northern analysis showed a 3.0-kb transcript for the EGFR extracellular domain motif homologue in amastigotes, but not in trypomastigotes. Binding of EGF to amastigotes induced signal transduction events. EGF induced *in vitro* kinase activity as determined by γ-[^{32}P]-ATP incorporation into amastigote proteins. EGF also increased protein kinase C activity and MAP kinase activity in the time- and concentration-dependent manner. A specific inhibitor (AG14782) of the EGFR and a MAP kinase inhibitor (PD98059) decreased the EGF-dependent *T. cruzi* MAP kinase activity. These results describe a novel mechanism used by amastigotes to regulate their proliferation mediated by an EGF-dependent signal transduction pathway.

The obtained data indicate a stimulating role of EGF in growth of trypomastigotes via activation of EGFR-like proteins and pose a question about its principal significance in signaling in these protozoa.

Thus, by the present time, proofs have been accumulated for the mitogenic action of mammalian growth factors on unicellular eukaryotes. Several protozoa have been found to have receptor-like proteins that have a structural similarity with growth receptors from mammalian cells. Proteins with tyrosine kinase activity have been revealed. The MAP kinase activated by human EGF has been detected. This proves the existence in several protozoa of signal pathways similar functionally with mammalian Ras-MAPK pathways. Their biological effect seems to be mediated by specific membrane sensors that have structural homology with mammalian growth factor receptors. Further study of molecular organization of the receptor-like molecules and their activated signal proteins as well as determination of induced genes is certainly to lead to a better understanding of mechanisms of action of mammalian growth factors on unicellular eukaryotes.

Phosphotyrosine-containing proteins and tyrosine kinases in unicellular eukaryotes

Cytoplasmic targets of action of protein tyrosine kinases in the lower eukaryote cells are numerous low-molecular proteins, the function of most of them yet remaining non-elucidated. Thus, according to data of phosphoamino acid analysis, insulin induces in the lower fungus *Neurospora crassa* phosphorylation of 14 proteins at serine, threonine, and tyrosine residues (Kole and Lenard, 1991). In solubilized membrane *N. crassa* fraction, insulin stimulated phosphorylation of 6 proteins at serine and tyrosine residue. A phosphotyrosine-containing protein of 38 kDa, pH 7.0–7.2, reacted at both immunoblotting and immunoprecipitation with antiserum to P2, a peptide from the human insulin receptor that contains autophosphorylated tyrosine residue. Tyrosine kinase activity was revealed in phosphoprotein of a molecular mass of 50 kDa when histone H2 was used as substrate (Fawell and Lenard, 1988). In the slime mold *Dictyostelium discoideum*, phosphotyrosine-containing proteins of molecular masses of 205–220 kDa were revealed (Schweiger *et al*., 1990). The presence of phosphotyrosine in *D. discoideum* proteins was established both from [^{32}P]-phosphate incorporation *in vivo* and in cell lysates with the aid of monoclonal antibody to phosphotyrosine *in vitro*. In the process of the initial growth phase of *D. discoideum*, phosphotyrosine-containing proteins of molecular masses of 205–220, 107, and 60 kDa were found; phosphorylation at tyrosine of protein with a molecular mass of 60 kDa was very low in this development phase of the myxomycete life cycle. Throughout the entire period of the fungal spore development an intensive phosphorylation at tyrosine of proteins of molecular masses of 205–220 and 107 kDa was observed; it decreased by 22 hr when the cell differentiation of *D. discoideum* spores was completed. At the same time, phosphorylation of tyrosine-containing proteins of a molecular mass of 60 kDa rose significantly in the differentiation phase and remained elevated during the whole period of the fruiting body formation; this indicates an important role of various tyrosine-containing *D. discoideum* proteins in the process of growth and differentiation. Recent investigations of effect of activated RasG on the phosphorylation state of *D. discoideum* proteins have shown that out of 70 vegetative phosphoprotein components resolved by two-dimensional (2-D) immunoblot analysis, three components were phosphotyrosine proteins (Secko *et al*., 2004). These data directly indicate participation of phosphotyrosine proteins in dictyostelium PTK-signaling.

By identification of phosphoamino acids in immunoprecipitation reaction the phosphotyrosine-containing proteins were revealed to contain, apart from phosphotyrosine, also phosphoserine and phosphotreonine. Studies with renatured proteins showed that, like RTK, at least one tyrosine kinase of *D. discoideum* was capable for autophosphorylation (Schweiger *et al*., 1990). The authors suggested that the phosphotyrosine-containing protein of a molecular mass of 60 kDa that is present in many multicellular animals could be related to a product of the gene *c-src*, while proteins forming bands in the area of 200–300 kDa could be receptor-like tyrosine kinases involved, like in the higher eukaryotes, in control of proliferation and differentiation (Table 1). The presence of receptor-like tyrosine kinases in *D. discoideum* was confirmed in the work of Adler *et al*. (1996). Based on results of molecular cloning and phylogenetic analysis, two kinases, DPYK3 and DPYK4, were concluded to belong to this RTK group. COOH-terminal fragments of each kinase are capable for autophosphorylation at tyrosine residues. These fragments were recognized in immunoblots by mAb 5E2 – monoclonal antibody to phosphotyrosine. However, analysis of the primary structures showed these enzymes, unlike classic RTK, to contain neither transmembrane segments nor SH2 domains. Like in RTK, in DPYK3 the domain I was functioning (from Lys-1050 to Met-1304), as well as possibly the more truncated domain II (from Thr-812 to Leu-1008). In DPYK4, only the domain I belongs to the tyrosine kinase branch of protein kinases. The tyrosine kinase ZAK1 (Table 1) is a downstream target of serpentine receptor cAR1 (Kim *et al*., 1999). It is required for GSK3 activation during multicellular development of *D. dictyostelium.*

Tyrosine kinases and phosphotyrosine-containing proteins were also revealed in various representatives of protozoa. Thus, tyrosine kinase-like activity was found in trophozoites and schizonts of *Plasmodium falciparum*; an inhibitory effect of the antimalarian drug piceatannol on

Table 1. Characteristics of tyrosine kinases from unicellular eukaryotes

		Properties of tyrosine kinases				
Species of the organism	Name of kinase	Size, kDa	Peculiar properties	Names of substrates	Function	Reference
D. discoideum	PTKs	60, and about 200 to 300	The presence of extracellular domain with the RTK activity is proposed	Not identified	Control of multicellular development	Schweiger *et al*., 1990
D. discoideum	DPYK3 DPYK4	150 75	C-terminal fragments of each protein are shown to be autocatalytically phosphrylated at tyrosine residues. The common feature of these kinases is the presence of two different sequence stretches in tandem that are related to kinase catalytic domains	Not identified	Not established	Adler *et al*., 1996
D. discoideum	ZAK1	No data	Downstream of GPCR	GSK3	ZAK1 phosphorylates and activates GSK3 for cell fate specification	Kim *et al*., 1999
D. discoideum	JAK-like kinase	No data	Activates STAT	DdSTAT	JAK/STAT regulates development of stalk cells during the multicellular part of the life cycle. Participates in control of cell movement	Dearolf, 1999; Hou *et al*., 2002
D. discoideum	PTK	No data	No data	PLA2	Through PLA2 activation can regulate Ca^{2+} influx	Schaloske and Malchow, 1997
L. donovani	PTK	About 110	No data	No data	Initiates host cell invasion	Salotra *et al*., 2000
L. mexicana	PTK	65	Upon IGF-I stimulation the receptor undergoes autophosphorylation at tyrosine residues	No data	Initiates host cell invasion	Gomes *et al*., 1998, 2001
P. falciparum	PTK	No data	The PTK activity associated with cell membrane. Chloroquine and piceatannol inhibited the PTK activity	No data	PTK is implicated in growth and parasite maturation	Sharma and Mishra, 1999; Mishra *et al*., 1999; Sharma, 2000; Sharma *et al*., 2000
T. thermophila	PTK	No data	PTK inhibitors blocked chemoattraction	No data	PTK is implicated in chemoattraction	Leick *et al*., 1997
C. reinhardtii	PK of the mixed type	48	PK is capable for autophosphorylation at serine and tyrosine	No data	PK is implicated in fertilization	Kurvari *et al*., 1996; Shiu and Li, 2004
N. fowleri	PTK	No data	PTK inhibitors blocked the proliferation	No data	PTK is implicated in growth	Chu *et al*., 2000
E. histolytica	c-Src	60	PTK is activated by FN binding with β1*Eh*FNR	Paxillin	PTK activates formation of signaling complex	Talamas-Rohana *et al*., 1998; Hernandez-Ramirez *et al*., 2000; Sengupta *et al*., 2000, 2001; Flores-Robles *et al*., 2003
E. histolytica	FAK-like kinase	125	Is activated by collagen type I and Ca^{2+} stimulation	Presumably the STATs	Is involved in transduction of invasive signal	Cruz-Vera, *et al*., 2003
T. cruzi	PTK	175	PTK is activated by stimulation of membrane gp82	No data	Initiates host cell invasion	Yoshida *et al*., 2000
T. brucei brucei	PTK	No data	The PTK activity is stimulated by IFN-γ	No data	Stimulates proliferation	Mustafa et. al., 1997

this enzyme was shown (Mishra *et al*., 1999). At present it is known that these kinases as well as trypanosomatid kinases similar functionally with mammalian tyrosine kinases are not true tyrosine kinases. This conclusion is made based on analysis of kinomes of these protozoa. It is established that no genes coding PTK are present in their genomes (Ward *et al*., 2004). In these parasitic microorganisms, kinases similar functionally with mammalian tyrosine kinases are ascribed to the tyrosine-like group that includes enzymes related to those in the PTK, although they are serine-threonine protein kinases (Ward *et al*., 2004).

In the same way, stimulation of parasitic ameboflagellates *Naegleria fowleri* by human serum increased ~~of~~ tyrosine phosphorylation in proteins of relatively low molecular mass (20, 22, 47, 51, and 53 kDa) and decreased phosphorylation of proteins of higher molecular masses (70–250 kDa) (Chu *et al*., 2000). The increase of tyrosine kinase activity in the *Naegleria fowleri* cell lysates was observed after 5 min, while the maximal phosphorylation of proteins at tyrosine, 15 min after addition of serum. Pretreatment of cell lysates with the inhibitor of tyrosine kinases genisteine significantly decreased phosphorylation *in vitro* of proteins at tyrosine (Chu *et al*., 2000). The importance of tyrosine phosphorylation during the life cycle of *Trypanosoma brucei* was proved for the first time by Marilyn Parsons and colleagues (Parsons *et al*., 1990; 1993). The authors demonstrated the appearance of tyrosine-phosphorylated proteins at 40–42 kDa during transition from slender to stumpy bloodforms. Other data indicate that phosphotyrosine-containing proteins with molecular masses of 34, 50, 82, and 121 kDa also occur in *T. brucei* (Mustafa et. al., 1997). Their phosphorylation was induced by interferon IFN-γ (100 units/ml) stimulation. IFN-γ induced a rapid and strong increase of tyrosine phosphorylation of several cellular proteins, which reached maximum after 5 min and was followed by a decrease to control levels after 120 min. *In vitro* application of tyrosine kinase inhibitor tyrphostin A47 at a non-toxic concentration of 1 μM to the trypanosome cultures caused a significant reduction of [^{3}H]thymidine uptake by IFN-γ-stimulated trypanosomes. In animals, 1 mg of the intraperitoneally injected tyrphostin A47 markedly reduced the parasite growth as compared with the vehicle dimethyl sulfoxide or the inactive compound tyrphostin A1. In conclusion, tyrosine kinases are strongly up-regulated in IFN-γ-stimulated *T. brucei*, and specific tyrosine kinase inhibitors can prevent trypanosome growth *in vitro* and *in vivo* (Mustafa et. al., 1997).

Yoshida et. al. (2000) reported involvement of *Trypanosoma cruzi* tyrosine kinase in the signal cascade that is initiated by a stimulation of glycoprotein gp82 and leads to mobilization of Ca^{2+} necessary for the onset of invasion. It was also found that the tyrosine kinase activity measured by the level of phosphorylation of protein of a molecular mass of 175 kDa (p175) was induced in metacyclic *T. cruzi* forms by extracts of HeLa cells, in which parasites develop effectively, but not by extracts of K562 cells resistant to invasion. Treatment with genistein blocked phosphorylation of protein p175 and increased cytosol Ca^{2+} concentration.

In promastigotes *Leishmania donovani*, phosphorylation at tyrosine of proteins with molecular masses of 105 and 110 kDa occurred at the first stage of invasion of the host cells. The heat shock induced by an elevation of temperature from 24°C to 37°C decreased the protein phosphorylation level at tyrosine in virulent, but not in avirulent parasite forms. This can indicate an important role of tyrosine phosphorylation in regulation of signal transduction at the initial stage of the host cells invasion (Salotra *et al*., 2000).

Tyrosine, serine, and threonine phosphorylation was studied in pro- and amastigote *Leishmania mexicana* forms, which was induced by insulin-like growth factor I (IGF-I) (Gomes *et al*., 1998). The phosphoamino acid analysis of patterns of the total and specific phosphorylation at tyrosine residues with use of labeled [^{32}P]-orthophosphate and immunoblotting with monoclonal anti-phosphotyrosine (anti-p-tyr) antibodies showed that stimulation of promastigote parasite forms with IGF-I induced tyrosine phosphorylation in protein of a molecular mass of 185 kDa, while stimulation of amastigote forms — phosphorylation at tyrosine of 40 and 60 kDa proteins. Analysis of total amino acid phosphorylation revealed additional phosphoprotein sets: protein of a molecular mass of 110 kDa in promastigotes and proteins of molecular masses of 120 and 95 kDa in amastigotes. Besides, stimulation of the cells with IGF-I and stage-specific changes in levels of the total phosphorylation and of phosphorylation at tyrosine were found in leishmania throughout the entire life cycle. This indicates an important role of tyrosine phosphorylation in regulation of the leischmania cell morphogenesis.

In *Entamoeba histolytica*, a membrane protein of a molecular mass of 220 kDa that had high level of tyrosine phosphorylation after interaction of *E. histolytica* trophozoites with fibronectin was revealed (Hernandez-Ramirez *et al*., 2000). Earlier, these authors characterized protein of a molecular mass of 140 kDa with the features of a β-1 integrin receptor that is associated with non-receptor tyrosine kinases (Table 1) and collagen-binding protein (Talamas-Rohana *et al*., 1998). The presence of tyrosine kinases in amoebae was confirmed in immunoprecipitation reaction with a combined use of the specific substrate peptide RR-SRC, tyrosine kinase inhibitor genistein, and monoclonal anti-p-tyr antibodies (Hernandez-Ramirez *et al*., 2000; Sengupta *et al*., 2000, 2001). Interestingly, an amoeba non-receptor tyrosine kinase (pp^{125}) is responsible for the amoeba STATs phosphorylation in response to collagen and calcium in *E. histolytica* trophozoites (Cruz-Vera *et al*., 2003). STAT amoeba homologues are involved in membrane-to-nuclei signaling and their tyrosine phosphorylation is essential for DNA binding and transcriptional enhancement.

Roisin *et al*. (2000) reported participation of tyrosine kinases in signal transduction in the protozoan parasite *Toxoplasma gondii*. The immunoblot analysis of tachyzoites lysate with anti-p-tyr 4G10 antibodies revealed tyrosine phosphorylation of several proteins in the range of 100–30 kDa. Phosphorylation of the 47 and 43 kDa proteins was found to occur only after the Ca^{2+} release. When the tachyzoites were stimulated by 50 mM K^{+} that promotes Ca^{2+} influx into the cells, increased

phosphorylation of two bands with an apparent molecular mass of 43 and 47 kDa was observed. This work also was the first to experimentally substantiate the role of dual phosphorylation at tyrosine and threonine within microbial protein tyrosine kinases TXY sequence (like ERK1 and ERK2) after tyrosine phosphorylation stimulation by Ca^{2+} (Roisin et. al., 2000).

Whereas structure and functions of specific mammalian tyrosine kinases have been studied sufficiently well, information about structure and properties of PTK in unicellular organisms is significantly less complete. The biological role of tyrosine kinases in unicellular eukaryotes also seems less clear than in mammalian cells. The unicellular eukaryotes, whose genomes were sequenced, have no receptor tyrosine kinases, i.e. there is no EGFR homologue, IGFR homologue in yeast, trypanosomatids, and Malaria parasites. Similarly, most protists lack true cellular tyrosine kinases. They have the tyrosine-like group, that includes enzymes related to those in the PTK, although they are serine-threonine protein kinases.

The role of the Ras subfamily proteins in signal transduction in unicellular eukaryotes

The Ras subfamily proteins are small monomeric GTP-binding proteins that regulate signal transduction pathways activated by RTKs. The main function of Ras subfamily proteins in signaling in metazoan cells consists in triggering the pathway that controls growth and neuronal development in mammalian cells, eye development in *Drosophila*, and vulva development in *C. elegans* (Kayne, Sternberg, 1995; Wassarman *et al*., 1995). In these cascades, the Ras protein interacts directly with Raf kinase, one of the large group of MAP kinase kinases or MEKKs (Fig. 1). However, at present there is convincing evidence for interaction of Ras with a number of other downstream effectors (Shields *et al*., 2000), including phosphoinositide-3-kinase (PI3K) and Ral-GDS, a guanine nucleotide exchange factor (GEF) for Ral, another member of the Ras subfamily of proteins. Furthermore, it is now clear that signals are transmitted to the Ras-GEFs not only through the RTKs, but also through heteromeric G protein-coupled receptors (GPCR) (Reuther, Der, 2000).

In mammalian cells, all RTK ligands and receptors are coupled to activation of the Ras-MAPK pathways through recruitment of Grb2 and/or Shc and the Grb2-bound exchange factor Sos. The main adaptor protein Grb2 has SH2- and SH3-noncatalytic domains of Src-homology, which recognize phosphorylated tyrosine in growth factor receptors and signal molecules. The guanine nucleotide exchange factor Sos activates Ras by exchanging its GDP for GTP (Egan, Weinberg, 1993). Activated Ras is bound to the serine threonine kinase Raf-1 (MEKK), activates it, and thereby triggers the MAP kinase cascade (Moodie *et al*., 1993).

Most Ras proteins revealed in unicellular eukaryotes belong to the Rab and ARF subfamilies of the Ras family proteins and do not participate directly in the PTK-mediated signal transduction. On the other hand, few members of the Ras subfamily have been shown to be present in fungal and protozoan organisms and to be highly conserved.

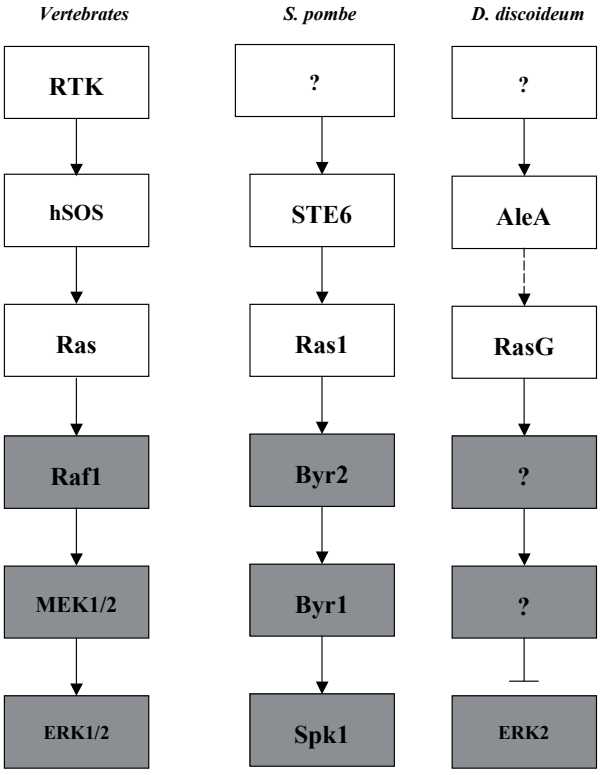


Fig. 1 Scheme of signal transduction via Ras-MAP kinase cascades in cells of vertebrates and unicellular eukaryotes. RTK- receptor tyrosine kinases; FAK– cytoplasmic tyrosine kinase; "?" – unidentified upstream or downstream targets within MAPK cascades; hSOS, STE6, AleA – guanine nucleotide exchange factors; Ras, Ras1, RasG – isoforms of small G-protein of the Ras protein subfamily; Raf1, Byr2 – isoforms of MAPKKK; MEK1/2, Byr1 – isoforms of MAPKK; ERK1, ERK2, Spk1 – isoforms of MAPK. *Gray color* indicates kinases of the MAPK signaling cascades.

Members of the Ras subfamily have been found in yeasts *Saccharomyces cerevisiae*, *Shizosaccharomyces pombe*, *Cryptococcus neoformans*, *Candida albicans*, *Ustilago maydis*, *Neospora crassa*, *Aspergillus nidulans*, slime mold *Dictyostelium discoideum* and *Physarum polycephalum*, protozoa *Entamoeba histolitica*, *Plasmodium falciparum*, and *Trypanosoma brucei* (Shen *et al*., 1994; Thelu *et al*., 1994; Kosaka *et al*.; 1998; Fronk, 1999; Sowa *et al*., 1999; Jaffer *et al*., 2001). Structure of a number of Ras proteins from fungi and protozoa was determined and their coding genes were cloned. However, biological role of most known microbial Ras subfamily proteins has not yet been determined sufficiently clearly, as in the majority of unicellular eukaryotes the intermediate and final chains of the signal pathways, in whose activation these proteins participate, are not yet identified. Ras proteins were found in yeast, slime mold, and protozoa, but functional studies are relatively scarce. The functions of the microbial Ras proteins are divided into three general categories: regulation of growth, regulation of differentiation, and regulation of the actin cytoskeleton, – functions that are conserved in metazoans (Lim *et al*., 2003; Weeks, Spiegelman, 2003). The structure of the Ras pathways in these unicellular eukaryotes vary from microorganism to microorganism and especially from the conventional metazoan Ras-MAP kinase pathways. It is

well known that in protists *Saccharomyces cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Ustilago maydis*, and *Dictyostelium discoideum* the Ras proteins activate adenylyl cyclase and that these cells expressing a constitutively activated Ras1 protein produce higher levels of cAMP (Toda *et al*., 1984; Alspaugh *et al*., 2000; Lim *et al*, 2001).

Since the characterization of the microbial Ras proteins has made an important contribution to understanding of Ras protein function in the lower eukaryote PTK-signaling; these studies will be summarized in this section (Weeks and Spiegelman, 2003).

The first microbial *ras* genes to be described were those from the budding yeast *Saccharomyces cerevisiae.* They express two *ras* genes, *ras1* and *ras2*, that encode proteins with nearly 90% homology to the first 80 positions of the mammalian Ras proteins, and nearly 50% homology to the next 80 amino acids (Powers *et al*., 1984). Monoclonal antibody against mammalian Ras proteins immunoprecipitated protein in yeast cells containing high copy numbers of the yeast *ras2* gene. The authors also showed that the yeast Ras proteins, like the proteins encoded by the mammalian *ras* genes, terminated with the sequence cysAAX, where A is an aliphatic amino acid. Thus, the yeast Ras proteins have the same overall structure and interrelationship as the family of mammalian Ras proteins (Powers *et al*., 1984). Functional studies demonstrated that yeast Ras proteins participated in regulation of growth (Tatchell *et al*., 1984; Wigler *et al*., 1988). *S. cerevisiae*, containing disruptions of either of two genes were viable, but haploid yeast spores carrying disruptions of both genes failed to vegetative grow (Kataoka *et al*., 1984; Tatchell *et al*., 1984). A loss of function mutation in either *ras1* or *ras2* has no effect on growth in glucose, whereas a loss of function of both genes (*rasl/ras2* null cells) causes arrest in the G_1 phase of the cell cycle (Tatchell *et al*., 1984). Ras2 is expressed in appreciably larger amounts than Rasl (Mosch *et al*., 1999) and overexpression of Ras1 completely suppresses the failure of *ras2* null cells to grow on non-fermentable carbon sources (Tatchell *et al*., 1985). The Ras proteins activate adenylyl cyclase and cells expressing a constitutively activated Ras protein produce higher levels of cAMP (Toda *et al*., 1985). The regulation of adenylyl cyclase by Ras involves both its direct binding to the enzyme and the participation of the adenylyl cyclase-associated protein, Srv2p (Shima *et al*., 2000). Ras is activated by the GEF, Cdc25, with *cdc25* null cells that have the same defective growth phenotype as *ras1/ras2* null cells (Robinson *et al*., 1987) and are inactivated by the GTPase activating proteins (GAPs), Ira1 and Ira2 (Tanaka *et al*., 1990). In *S. cerevisiae* cAMP activates the cAMP dependent protein kinase A (PKA) and *rasl/ras2* null strains are suppressed either by a disruption in the gene encoding the negative regulatory subunit of PKA or by overexpression of a catalytic subunit of PKA. In addition to regulating progression through G_1 the Ras-PKA pathway is a negative regulator of the stress response. Thus, for example, cells expressing an activated *ras* allele are less successful at surviving nitrogen starvation or heat shock (Kataoka *et al*., 1984, Nikawa *et al*., 1987). Recently, several new targets of the stress-activated Ras-PKA pathway have been discovered. These include the Msn2 and Msn4 transcription factors mediating a part of induction of STRE-controlled genes by a variety of stress conditions, the Rim15 protein kinase involved in stationary phase induction of a similar set of genes and the Pde1 low-affinity cAMP phosphodiesterase that specifically controls agonist-induced cAMP signaling (Thevelein and Winde, 1999).

Diploid *S. cerevisiae* exhibit filamentous growth (pseudohyphal growth) in response to either nitrogen starvation or an excess of a fermentable carbon source (Gimeno *et al*., 1992). The *ras2* null cells lose the filamentous growth phenotype and two parallel signaling pathways that involve Ras2 have been implicated. One of these is a MAP kinase cascade that uses some of the kinase components responsible for Ras-independent haploid cell mating. However, during pseudohyphal growth, the MAP kinase cascade in *S. cerevisiae* is triggered by signals transmitted from Ras through the RHO (Rho) subfamily protein Cdc42, either of two 14–3-3 proteins, Bmhl and Bmh2, and the Ste20 protein kinase (Mosch *et al*., 1996; Mosch and Fink, 1997; Roberts *et al*., 1997). It is interesting that in mammals the GTP-bound Rho also can regulate the stress-activated p38- and JNK-MAPK pathways. The latter pathway that regulates pseudohyphal growth involves a GPCR, Gprl, a $G\alpha$ protein, Gpa2, Ras, ACA, and PKA (Kubler *et al*., 1997; Lorenz *et al*., 2000). Activation of PKA by mutations in the *bcy1* gene that encodes the PKA regulatory subunit induces filamentous growth in both wild type cells and in strains deficient in components of the MAP kinase pathway (Pan and Heitman, 1999; Rupp *et al*., 1999). These results indicate that PKA activation is sufficient to trigger filamentous growth.

Ras2 is also essential for the haploid invasive growth that occurs after prolonged incubation on rich media, possibly in response to metabolic by-products (Mosch *et al*., 1999; Stanhill *et al*., 1999). Overexpression of Rasl can rescue the invasive growth defect of haploid *ras2* null cells, indicating that there is insufficient Rasl present in a *ras2* null cell lo allow invasive growth (Mosch *et al*., 1999). Haploid invasive growth can be induced by either the Ras dependant MAP kinase cascade or the Ras dependent PKA pathway (Mosch *et al*., 1999; Roberts and Fink, 1994).

S. cerevisiae also has a single *ras*-related *rap* gene, *rsr1*, and its deletion disrupts the normal pattern of bud site selection during normal haploid growth (Bender and Pringle, 1989). Rsr1 is highly concentrated at the incipient bud site in the plasma membrane and is essential for the co-localization of Cdc42 (Park *et al*., 2002). Although the Ras proteins and Rsr1 clearly have independent functions, Rsr1 is capable of substituting for Ras function when overexpressed in *ras1/ras2/cyr1* null cells. It has been speculated that, in addition to Ras2, Cdc42 and Bmh1/2 could also be involved in regulation of cytoskeletal polarity during temperature stress in haploid cells (Ho and Bretscher, 2001).

The Ras-mediated MAP kinase cascade also has been described in yeast *S. pombe*, C. *albicans*, and *C.*

neoformans and possibly in *U. maydis* and *S. cerevisiae* (Zhang *et al*., 2001; Weeks, Spiegelman, 2003). However, at present there is evidence that only in *S. pombe* Ras-MAPK the pheromone response pathway is clearly analogous to the Ras-activated MAP kinase cascade in mammalian cells (Hughes *et al*., 1993). In the *S. pombe*, Ras1 is activated by stimulation of the GEF activity (STE6), this resulting in the consecutive activation of Byr2 (MEKK), Byr1 (MEK), and Spk1 (MAP kinase) (Fig. 1). The Byr1 is dual protein kinase that phosphorylates Spk1 at both tyrosine and threonine residues in the subdomain VIII of the protein. Phosphorylation of both amino acids has been shown to be essential for Spk1 function. In turn, action of this kinase is to activate phosphorylation transcription factors, which leads to altered gene expression.

Ras1 may also stimulate a signal pathway involved in cell morphogenesis (Chang *et al*., 1994; Hughes, 1995). The *ras1* null cells *S. pombe* exhibit abnormal cell shape and the absence of polarized cell growth, phenotypes shared with *scd1* mutants. Ras1 interacts with Scd1, a putative GEF for Cdc42 (Chang *et al*., 1994). Since *ras1* null cells exhibit disorganized cytoskeletal structures, it has been speculated that Ras1 is a part of the complex that regulates actin and microtubule assembly (Hughes, 1995). Two-hybrid interaction analysis has implicated downstream components of the Ras1-Scd1 pathway in regulation of formation of the mitotic spindle, a microtubule structure (Li *et al*., 2000). The second RasGEF, Efc25, has recently been characterized (Papadaki *et al*., 2002). This protein also regulates the Ras1-Scd1 pathway, but not the Ras1-Byr2 MAPK pathway. Thus, in the *S. pombe* three GEFs, STE6 and Scd1 or Efc25, activate two these distinct downstream pathways regulated by Ras1.

Ras genes were isolated from the slime mold *Physarum polycephalum* (Kozlowski *et al*., 1993), *Dictyostelium minutum* (Van Es *et al*., 1997) and *Dictyostelium discoideum* (Reymond *et al*., 1984, Robbins *et al*., 1989, 1990; Daniel *et al*., 1994). The Ras proteins play an importance role in the life cycle of these microorganisms, but functional studies have been performed only on *D. discoideum*; so only the Ras proteins of this latter species will be discussed.

In the *D. discoideum* six *ras* subfamily genes, *rasG*, *rasD*, *rasB*, *rasC*, *rasS* and *rapA*, have been thus far partially characterized (Reymond *et al*., 1984; Daniel *et al*., 1994). Only two of the encoded products, RasG and RasD, are highly related to each other and are the *D. discoideum* proteins most related to mammalian Ras. RasB. RasC and RasS are somewhat divergent from each other and from RasG and RasD (Daniel *et al*., 1994). The Rapl protein is 76% identical to human Rapl, suggesting that they are homologues.

rasD, the first *Dictyostelium ras* gene to be described, was originally isolated as a prestalk cell enriched mRNA species from developing cells (Reymond *et al*., 1984). It is expressed at very low levels in vegetative cells, but expression increases dramatically during late aggregation. RasG was isolated from a clonal library, using the *rasD* gene as a probe (Robbins *et al*., 1989). It is expressed during growth and early development, but expression declines rapidly as development progresses. The expression of activated rasG in prespore cells results in their transdifferentiation into prestalk cells (Jaffer *et al*., 2001).

Transformants overexpressing a consiitutively activated form of RasD, from the *rasD* promoter, produce multi-pletipped aggregates that do not develop further (Weeks and Spiegelman, 2003). These transformants exhibit enhanced expression of prestalk cell specific genes (Louis *et al*., 1997). Surprisingly, *rasD* gene disruption caused no change in cell type determination (Wilkins *et al*., 2000). The only observable defect of *rasD* null strains is a near total loss of the phototactic and thermotactic properties of the slugs. Since *rasD* expression is enriched in the prestalk cell populations in the anterior region of the slug (Esch and Firtel, 1991), it is plausible that RasD is involved in modulation of sensory signaling from the photo- and thermoreceptors that are presumably localized at the anterior tip of the slug (Fisher, 1997). Although *rasG* expression decreases during development, there are still appreciable levels of the protein present during aggregation and it is possible that RasG substitutes for cell type determination functions of RasD in *rasD* null cells.

RasG is the major Ras species in vegetative cells *D. discoideum* and expression of constitutively activated RasG during vegetative growth results in cells that fail to aggregate, due to a reduced capacity to generate cAMP (Khosla *et al*., 1996), *rasG* gene disruption results in cells that exhibit a complex phenotype. Vegetative *rasG* null cells have aberrant lamellipodia, multiple elongated filopodia, and unusual punctate polymerized actin structures (Tuxworth *et al*., 1997). The cells exhibit reduced motility and polarity. They grow more slowly and to a much lower final density than the parental wild-type cells in suspension culture and are multinucleate, suggesting a problem in cytokinesis. It is interesting that both the disruption of the *rasG* gene and the expression of constitutively active RasG inhibit growth, motility, and chemotaxis, suggesting that a precise level of signaling is necessary (Tuxworth *et al*., 1997).

In *rasG* null cells, levels of RasD protein are substantially increased and expression of RasD under the control of the rasG promoter rescues all adverse consequences of *rasG* gene disruption, except for the motility defect (Khosla *et al*., 2000). The combined loss of RasG and RasD may be lethal, since attempts at isolating *rasG/rasD* double gene disruptants have been unsuccessful. RasG appears to negatively regulate vegetative cell expression from several of the *ras* gene promoters. Thus, the levels of the various Ras proteins are tightly regulated in vegetative cells *D. dictyostelium* (Khosla *et al*., 2000).

RasG appears to negatively regulate ERK2, a MAP kinase that is activated in response to cAMP and is required for accumulation of cAMP (Segall *et al*., 1995). The cAMP stimulation of phosphorylation of ERK2 is enhanced in *rasG* null cells and reduced in cells overexpressing activated RasG (Kosaka *et al*., 1998). Cells lacking ERK2 fail to aggregate (Segall *et al*., 1995; Wang *et al*., 1998) and a disruption of the *regA* gene, encoding a phosphodiesterase, rescues this defect. A plausible explanation for these results is that phosphorylated ERK2 inhibits the phosphodiesterase activity of RegA (Shaulsky *et al*., 1998), thereby resulting in cAMP accumulation and

PKA activation (Loomis, 1998). In cells overexpressing activated RasG, a reduction in phosphorylated ERK2 could result in insufficient cAMP accumulation to fully activate PKA, *erkB* null cells form few spores, suggesting a requirement for ERK2 for the expression of prespore cell specific genes (Gaskins *et al*., 1996) and the developmental expression of activated RasD also results in down-regulation of prespore cell specific gene expression (Louis *et al*., 1997; Jaffer *et al*., 2001).

RasC is necessary for cAMP relay during aggregation *D. discoideum*, so that the activation of adenylyl cyclase (isoform ACA), in response to cAMP, is reduced to a negligible level in *rasC* null cells (Lim *et al*., 2001). However, addition of GTPγS to extracts of *rasC* null cells, which bypasses the need for cAMP binding to cARl, results in a partial stimulation of ACA activity (Lim *et al*., 2001). These results place RasC upstream of Gα2βγ in mediating activation of ACA. Analysis of the cAMP binding to cell membranes following GTPγS addition, as a measure of Gα2βγ dissociation (Kumagai *et al*., 1991), is consistent with the idea that RasC may be involved in regulating Gα2βγ dissociation (Weeks and Spiegelman, 2003).

In addition to its requirement for cAMP relay, RasC is also clearly involved in regulation of vegetative growth (Kae *et al*., 2004) and chemotaxis (Wessels *et al*., 2004). During the early phase of aggregative development, *rasC* null cells exhibit reduced polarization and chemotaxis poorly in a spacial cAMP gradient relative lo wild type cells, whereas cAMP pulsed *rasC* null cells exhibit increased polarization and chemotaxis more rapidly relative to wild type cells (Lim *et al*., 2001). However, despite this apparent enhanced chemotactic ability in a spatial cAMP gradient, these cells do not respond to a simulated temporal gradient of cAMP.

The phosphorylation of protein kinase B (PKB) and its polarized translocation to the membrane in response to cAMP has been implicated in the chemotactic response (Meili *et al*., 1999; Firtel and Chung, 2000). Although PKB phosphorylation is significantly reduced in *rasC* null cells upon cAMP stimulation (Lim *et al*., 2001), PH-domains of PKB are successfully translocated to the plasma membrane of cAMP stimulated *rasC* null cells. It appears, therefore, that RasC is not necessary for generation of PH-domain recruitment sites, although a contributory role cannot be totally ruled out. Since PKB phosphorylation in response to cAMP is also reduced in *rasG* null cells and RasG interacts with PI3-kinase in a yeast two hybrid assay (Lee *et al*., 1999), it is possible that both RasC and RasG independently contribute to the regulation of PKB activity and translocation, via the PI3-kinases. Mutants disrupted in both *rasC* and *rasG* might exhibit a more severe chemotaxis defect.

Possible signaling partners of RasG and RasC include RIP3, identified as a Ras-interacting protein in a yeast two-hybrid screen (Lee *et al*., 1999), and the RasGEF AleA (Insall *et al*., 1996; Lim *et al*., 2001). Both *rip3* and *aleA* null cells exhibit defects in cAMP relay and there is a partial stimulation of ACA activity in their cell lysales (Lee *et al*., 1999; Lim *et al*., 2001), suggesting the coordinate involvement of RasC, Rip3, and AleA in a pathway that regulates Gα2βγ function. ERK2 phosphorylation is upregulated in response to a cAMP stimulus in *aleA* null cells (Aubry *et al*., 1997; Lim *et al*., 2001), a response similar to that observed in *rasG* null cells. It is possible, therefore, that AleA and Rip3 regulate activities of both RasC and RasG.

Disruption of the *rasS* gene results in cells that fail to grow under axenic conditions, which correlates with a dramatic impairment in fluid phase endocytosis and a reduced number of the crown-like morphological structures that mediate macropinocytosis (Chubb *et al*., 2000). These *rasS* null cells also exhibit a three-fold enhancement of motility relative to the parental strain. It has been suggested that there is competition for the recruitment of cytoskeletal organizing proteins, such as coronin and myosin I between the endocytic cups and the pseudopodia, which would limit the ability of cells to stimulate both endocytosis and movement at the same time. Hence, RasS may regulate the balance between feeding and movement (Chubb *et al*., 2000). *rasS* null cells differentiate normally, indicating that RasS is not required for the differentiation process.

In protozoan organisms the Ras subfamily proteins were found only in *Entamoeba histolitica*, *Plasmodium falciparum*, and *Trypanosoma brucei*. In the *E. histolitica*, genes homologous to the *ras* and *rap (Krev-1*) were cloned (Shen *et al*., 1994). Two putative ameba *ras* genes (*Ehras1* and *Ehras2*) that contain 205 and 203 amino acid (aa) open reading frames (ORFs), respectively, were identified. The *Ehras1* ORF shows a 91% positional identity with that of *Ehras2*, a 55% identity with *Dictyostelium discoideum* (*Dd*) *ras*, and a 47% identity with human (*Hs*) *ras*. The *Ehrap1* ORF shows a 93% positional identity with that of *Ehrap2*, a 60% identity with *Dd rap*, a 61% identity with *Hs Krev-1*, and a 45% identity with that of *Ehras1*. Conserved aa in each ameba *ras* and *rap* ORF include GTP-binding sites, effector site, site of ADP-ribosylation by *Pseudomonas* exoenzyme S, and COOH-terminus CAAX. A single 21-kDa ameba Ras protein reacted with the rat Y13–259 anti-ras monoclonal antibody located on the cytosol side of the plasma membrane. These were the first *ras* and *rap* genes identified from protozoan parasites. A gene has been identified in the *T. brucei*, and the encoded product is equally related to the Ras and Rap proteins of *E. histolitica*. This suggests that this gene is possibly an evolutionary ancestor of both *ras* and *rap* genes of other eukaryotes (Sowa *et al*., 1999). The Ras proteins identified in protozoan microorganisms are present during all stages of the life cycle, but their role in signal transduction is not yet clear.

Thus, the signaling pathways regulated by Ras proteins in mammalian and microbial cells differ functionally and structurally. The most intensively studied microbial Ras subfamily members activate adenylyl cyclase and are involved in Ras-PKA pathways; only in few eukaryotic microorganisms, Ras proteins are involved in Ras-MAPK signaling that have great similarity with such in mammals. This suggests that in evolution of the eukaryotic organisms the Ras proteins originally participated in regulating cAMP-dependent signal pathways and their role in the MEKK-MEK-MAPK module stimulation is connected with transition from unicellularity to multicellularity.

The MAPK signaling cascades in unicellular eukaryotes

In mammalian cells, tyrosine phosphorylation is rapidly and sequentially translated to changes in phosphorylation of protein serine/threonine MAP kinases (MAP3Ks and MAP2Ks). The latter are highly specific for the downstream components ERK-1 and ERK-2 MAPKs. Activation of these kinases results from phosphorylation of threonine and tyrosine residues in a TXY-motif that is common to most MAPKs. MAP kinases activate several regulatory proteins in the cytoplasm and in the nucleus to initiate such cellular processes as proliferation, differentiation, and development (Seger and Krebs, 1995).

As soon as mammalian MAP2K (MEK) and MAPKs had been cloned, they were noticed to have sequence homology to yeast signal transduction kinases that are sequentially activated in response to extracellular stimuli. Later on, data appeared that the MAPK that have a TXY-motif were also present in other unicellular eukaryotes. In this connection, it is interesting to compare the microbial MAP kinase signaling cascades with those in vertebrates. This will allow better understanding the functional role of tyrosine phosphorylation in regulation of signal transduction MAP kinase pathways and specifying significance of individual MAPK components that are common to all eukaryotes.

In *Saccharomyces cerevisiae*, several different MAPK cascades exist, i.e. multiple sets of similar enzymes form distinct pathways that are activated by different external signals (e.g., mating pheromone, cell wall integrity, and osmolarity changes (Figs. 2 and 3). The diversity and function of MAPKs in yeast have been the subject of several excellent reviews (Errede and Levin, 1993; Neiman, 1993; Ammerer, 1994; Herskowitz, 1995; Elion, 2000). In the current review we will concentrate on the signaling pathways that use MEKK, MEK and yeast MAPK isoforms that appear to be the principal ones in mammalian growth factor signaling.

The yeast MEKK-MEK-MAPK cascades regulate at least six functionally different signal pathways. They govern transitions in its life cycle – mating and invasiveness in haploid strains and pseudohyphal development and spore formation in diploid strains – as well as maintenance of cells at a high osmolarity (Herskowitz, 1995). Studies of these different pathways provide a great amount of information on different possible inputs to MEKK–MEK–MAPK modules, from two-component system, SH3-domain sensor protein Sho1p, G protein-coupled receptors and PKC1 and to new components that play important roles

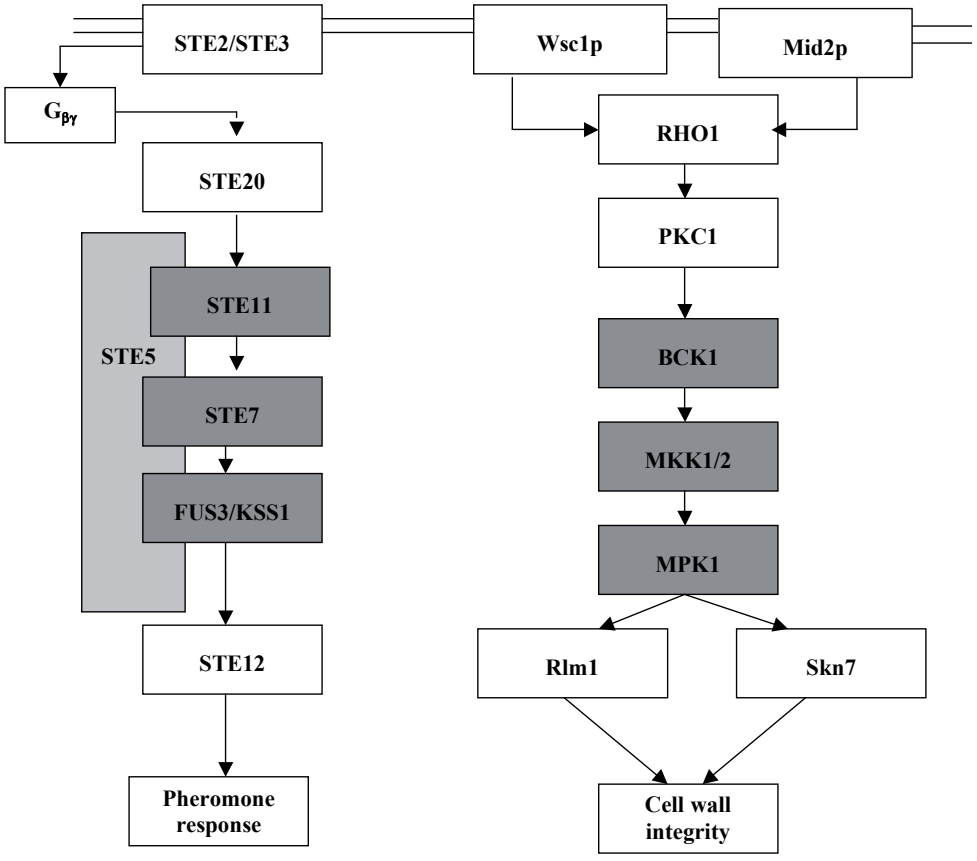


Fig. 2 The MAPK of *S. cerevisiae* activated by G-protein-coupled receptors STE2/STE3 and PKC1. STE2/STE3 – serpentine G-protein-coupled receptors for pheromones; Wsclp, Mid2p – membrane sensors; RHO1 – small G-protein; Gβγ (STE4) – βγ-subunit of heterotrimeric G-protein; STE5 – scaffold protein; STE20 – PAK-like kinase; STE11, BCK1 – MEKK homologues; STE7, MKK1/2 – MEK homologues; FUS3, KSS1, MPK1 – MAPK homologues; STE12, Rlml, Skn7 – transcription factors. Dark gray shading indicates kinases of the MAPK signaling cascades. Light gray shading indicates scaffold protein.

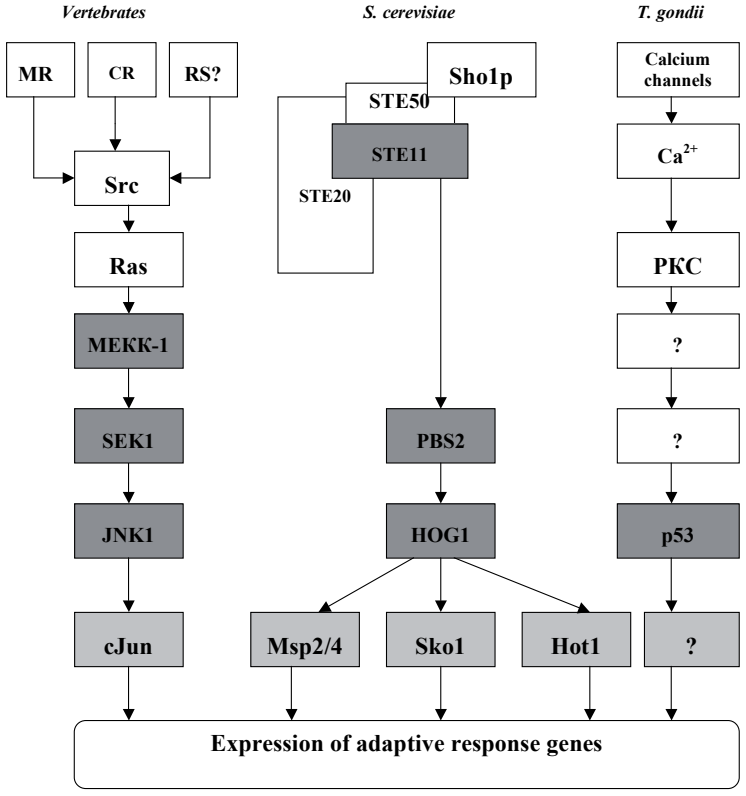


Fig. 3 Stress-activated MAP kinase cascades in vertebrates and unicellular eukaryotes. MR – mechanoreceptors; CR – cytokine receptors; RS – suggested redox-sensitive sensors; OS – suggested osmosensors; Src – non-receptor tyrosine kinases; PKC – protein kinase C; (?) – unidentified targets; Ca^{2+} – calcium ions; Ras – small G-protein; Sho1p – SH3-containing osmosensor protein; STE20 – PAK-like kinase; STE50 – subunit of STE11 kinase. MEKK-1, SEK1, JNK1, PBS2, HOG1, p53 – kinases of stress-activated MAP kinase cascades. c-Jun, Msp2/4, Sko1, Hot1 – factors of transcription. Taupe colour – kinases of the MAPK signaling cascades; light gray – transcription factors. Details in the text.

in the MEKK–MEK–MAPK module and are likely to be found in mammalian cells. Here we will consider only two MAP kinase cascades *S. cerevisiae*. One of them, the well–studied MAPK module of the pheromone response pathway is involved in mating and other steps in their yeast life cycle, and is interesting in that it is activated through serpentine G protein-coupled receptors STE2/STE3 by the mechanism that is partially similar to that in the higher eukaryote cells (Herskowitz, 1995). The other cascade is responsible for cell wall integrity and is activated by PKC1 (Blumer *et al*., 1994), as well as by several mammalian signaling cascades (Seger and Krebs, 1995). Only in the pheromone response pathway and in the PKC1 pathway the *S. cerevisiae* have complete MAPK cascades with identified MEKK, MEK, and MAPK.

Unlike mammalian, *Drosophila*, and nematode cell systems with their Raf-MEK-MAPK module, in which MEK activity can be stimulated by activating a RTK to lead to activation of the protein kinase Raf, this MEKK cascade activity in *S. cerevisiae* that lacks RTK and Raf is controlled ultimately by serpentine G protein-coupled receptors.

A typical example of signal transduction to the yeast MAPK cascade with participation of serpentine receptors can be the pheromone response pathway. This response pathway is necessary for haploid strains of yeast that are of the mating type a or α to mate with each other. There are three major responses: transcriptional induction of genes involved in mating, arrest of cells in G_1, and morphological changes. Each of the haploid cell types produces a peptide mating pheromone, a factor or α factor, that acts on the serpentine receptors of its mating partner. The a cells produce the α factor receptor STE3, whereas the a/α cells produce none. Synthesis of the receptor is governed at the transcriptional level by cell type-specific regulatory proteins; all intracellular components of the pheromone response pathway are common to the a and α cells (Herskowitz, 1995).

Activation of the receptor-caused dissociation of Gα from G$\beta\gamma$ of heterotrimeric G protein then activates the MAPK cascade in an as yet unknown manner involving STE20 and STE5. STE5 acts as a scaffold for the MAPK cascade and is not associated with Gβ (STE4), STE20 or STE12, but is associated with STE11 and STE7, as well as with MAPKs, FUS3, and KSS1. These results lead to suggestion that STE5 acts as a scaffold for the MAPK module (Fig. 2).

STE11, a MEKK family member, then phosphorylates and thereby activates STE7, a MEK family member (Neiman and Herskowitz, 1994). STE7, in turn, phosphorylates the MAPKs FUS3 and KSS1 and activates them (Gartner *et al*, 1992; Errede *et al*., 1993). The activated MAPKs then apparently activate the transcription factor, STE12, by phosphorylating it (Elion *et al*., 1993). STE12, often in association with the general transcription factor MCM1, then activates transcription of numerous genes coding for components of the pheromone response pathway itself and genes necessary for cell fusion. Cell cycle arrest in

response to the mating pheromones results ultimately from action of the FAR1 protein that binds to cycline-dependent kinases CDC28-CLN1 and CDC28-CLN2 to inhibit their activity (Peter and Herskowitz, 1994).

Biochemical studies have shown that FUS3, in addition to FAR1 and STE12, has several other substrates, including STE7 (Zhou *et al*., 1993) and STE5 (Kranz *et al*., 1994); however, their functional significance is unknown: they might play a role in down-regulation of the pathway or in activation of the pathway. Answer to this question needs further investigations.

Signal transmission from growth factor receptors in mammalian cells to the MAP kinase cascade might include not only participation of RTK and GPCR, but also PKC-activating mechanisms. These have not yet been sufficiently studied; they include multiple levels of a cross-talk existing between the both signal systems at early points during signal events. Therefore, it is particularly interesting to consider the direct way of the extracellular signal transmission with participation of PKC1 (isoform of PKC) onto the MAP kinase cascade (MEKK-MEK-MAPK) in yeasts.

In *S. cerevisiae*, like in some other eukaryotic organisms (Figs. 2 and 3), *PKC*1 participates in regulation of stress-activated signal transduction pathways. Mutants lacking *PKC1* (gene encoding yeast PKC1) at all temperatures has a cell lysis defect that can be suppressed by a high-osmolarity medium (Levin and Bartlett-Heubusch, 1992; Paravicini *et al*., 1992).

These components of the PKC1 pathway have been identified by isolation of supressors, in particular, the high copy plasmid suppressors that restore growth in mutants defective in different components of this pathway (Lee and Levin, 1992; Irie *et al*., 1993; Lee *et al*., 1993; Errede *et al*., 1995). These studies have led to identification of genes for a MEKK homolog, *BCK1*, a MEK homolog, *MKK1*, and a MAPK homolog, *MPK1.* The *MKK1* gene has turned out to be functionally redundant with a related gene, *MKK2* (Irie *et al*., 1993). Mutants lacking any of these components – the MEKK (*bck1*), two MEKs (*mkk1* and *mkk2*), or the MAPK (*mpk1*) showed the same phenotype: lysis at a high temperature. Since the phenotype of mutants deleted for PKC1 is more severe than for defective mutants, it is suggested that PKC1 controls a bifurcated pathway, one arm of which contains the MAPK cascade (Lee and Levin, 1992). Upstream components that could control PKC1 include the putative membrane sensors Wsc1p and Mid2p (Ketela *et al*., 1999) as well as the RHO1 small G-protein (Nonaka *et al*., Qadota *et al*., 1996) (Fig. 2).

By the present time it is established that the *S. cerevisiae* PKC1 is an activator of the MAP kinase cascades that perform transmission of signals inducing the cell processes that are aimed not only at maintenance of the cell wall construction (Lee, Levin, 1992; De la Torre-Ruiz *et al*., 2002; Krause and Gray, 2002; Reinoso-Martin *et al*., 2003), but also at polarized growth (Philip and Levin, 2001) and adaptation (Kamada *et al*., 1995, Ketela *et al*., 1999). Interestingly, in the closely related species *S. pombe* the Pkc1p and Pkc2p that have a high degree of structural homology with the *S. cerevisiae* PKC1 did not transmit signals to the MAP kinase cascade (Perez and Cthroughe, 2002). Both PKC forms in these yeasts also are involved in regulation of processes of cellular integration, but mediate their action through reaction of biosynthesis of the cell wall polymer (1→6)-β-glucan with participation of the enzyme (1–3)-β-D-glucansynthase (Arellano *et al*., 1999).

In other model eukaryotic microorganisms, no complete MEKK-MEK-MAPK modules have been revealed so far. However, they were found to contain integral MAP kinases phosphorylated at tyrosine and threonine, which indicates a great importance of the activated MAP kinases for down-regulation of cellular events.

In the slime mold *Dictyostelium discoideum* the MAP kinase ERK2 has been shown to be essential for growth and to play a role in multicellular development (Gaskins *et al*., 1994). Overexpression of ERK2 in *D. discoideum* results in abnormal slug morphogenesis and fruiting body formation. In addition, ERK2 in *D. discoideum* is important for receptor-mediated stimulation of adenylyl cyclase ACA and is required for accumulation of intracellular cAMP (Segall *et al*., 1995; Maeda *et al*., 1996; Aubry *et al*., 1997; Kuwayama *et al*., 2000). Activation mechanisms in MAPK signaling in *D. discoideum* are not yet completely clear. The cAMP acting through serpentine cAMP receptors is known to produce a rapid and transient stimulation of the *D. discoideum* ERK2 activity (Maeda and Firtel, 1997). This might indicate that the *D. discoideum* ERK2 can be activated by G protein-coupled receptors by the mechanism that is similar with that in the higher eukaryotes. The MAP kinase cascade in *D. discoideum* is a part of the complex signal pathway required for cell aggregation. This pathway includes RasGEF AleA and RasG (Fig. 1) that are important regulators of ERKs activation and adaptation. By examining both the level and kinetics of activation and adaptation of ERK2, it was shown that Ras was a negative ERK2 regulator. Activation of Ras or disruption of the RasGAP gene resulted in reduced ERK2 activation, whereas disruption of putative RasGEF or expression of dominant negative Ras proteins (which are described in detail in the preceding Section) have a more rapid, higher, and extended activation. Moreover, the constitutive expression of a PKA catalytic subunit was shown to bypass the requirement of ERK2 for aggregation and later development, indicating that PKA lies downstream from ERK2 and that ERK2 may regulate one or more components of the signaling pathway required for mediating the PKA function (Aubry *et al*., 1997).

Recently, MAP kinases and genes encoding microbial MAPK in parasitic fungi *Fusarum oxysporum* (Di Pietro *et al*., 2001), in protozoan organisms *Plasmodium falciparum* (Doerig *et al*., 1996; Lin *et al*., 1996; Graeser *et al*., 1997), *Toxoplasma gondii* (Ng *et al*., 1995, 1997; Roisin *et al*., 2000), *Tetrahymena thermophila* and *Tetrahymena pyriformis* (Nakashima *et al*, 1999), *Trypanosoma brucei* (Hua and Wang, 1994, 1997), and *Leishmania mexicana* (Wiese, 1998) have been identified. These kinases contribute to amplification and specificity of transmitted signals in the nucleus to initiate cellular processes, such as growth, development, and morphogenesis. However, identity of these kinases is not known. Certainly, they lack transcriptional regulation and the mechanisms, by which proliferation is regulated in protozoan and metazoan

cells, are different. Many of them do have multiple protein kinases similar to MAPKs, but for the most part their roles are not yet established.

MAP kinase of *T. gondii* has several forms (Roisin *et al*., 2000). It is interesting that when using antibodies to the mammalian ERK active forms recognizing only MAP kinases phosphorylated at tyrosine and threonine, not two activated MAP kinases usually detected by these antibodies, but six, of molecular masses of 43, 45, 47, 53, 67, and 80 kDa, were found in *T. gondii*. An immunoblot analysis with the use of anti-ERK1 and anti-ERK2 antibodies revealed several immunoreactive bands at 57, 53, 47, and 43 kDa. Based on similarity of molecular weights and kinase activities with mammalian MAP kinases, the p47 and p43 protein were identified as homologues of ERK1 and ERK2, respectively. The p53 protein revealed by specific anti-SAPK antibody can correspond to p54-JNK2/SAPK, while the p57 band detecting anti-ERK antibodies, but not forming immune complexes with antibody to the active ERK form might presumably be an inactive isoform of the ERK homologues (Roisin *et al*., 2000) or the MAP kinase that, like ERK3, is not activated by phosphorylation at tyrosine and threonine residues inside the TXY-motif (Boulton *et al*., 1991). The p67 band, phosphorylated myelin basic protein (MBP), was also detected with the anti-active ERKs antibodies, suggesting that this protein contains the TXY motif and seems to be an ERK7 homologue (Abe *et al*., 1999). The nature of the redox-sensitive p80 kinase of *T. gondii* has not been yet elucidated. The p80 protein detected using the anti-active ERK antibodies was activated in response to H_2O_2, which suggests that the p80 can be a homologue of the mammalian ERK5 (Abe *et al*., 1996). The large spectrum of the revealed MAP kinase isoforms in *T. gondii* indicates an important role of these enzymes in coordination of many cellular processes.

A MAPK homologue of *Plasmodium falciparum*, PfMAP (also known as Pfmap-1), was simultaneously discovered independently in three laboratories (Doerig *et al*., 1996; Lin *et al*, 1996; Graeser *et al*., 1997). It was shown that, like other MAP kinases, it contained a TXY-motif in the kinase subdomain VIII, whose phosphorylation at tyrosine and threonine is essential for activity (Robbins *et al*., 1993; Graeser *et al*., 1997). Use of immunoblot analysis and immunopreciptation reaction has allowed revealing in *P. falciparum* four different MAP kinase forms: p40, p80, p100, and p150 (Lin *et al*, 1996, Graeser *et al*., 1997). The total MAP kinase activities increased in the process of parasite maturation (Graeser *et al*., 1997). Activity of the p80 kinase was high at the asexual stage of parasite development, whereas the p100 protein activity, on the contrary, in gametes and zygote. It is yet to be elucidated whether the PfMAP kinase forms in *P. falciparum* are involved in control of growth and differentiation of the parasites or participate in different signal pathways and perform other cell functions (Kappes *et al*, 1999). Recently, another MAPK homologue was identified in gametocytes of *P. falciparum* (Dorin *et al*., 1999). It displayed functional characteristics of MAPKs such as (1) ability to undergo autophosphorylation, (2) ability to phosphorylate myelin basic protein, a classical MAPK substrate, (3) regulation of kinase activity by a MAPK-specific phosphatase, and (4) ability to be activated by component(s) present in cell extracts. But the peculiarity of Pfmap-2 is that it does not have the conserved threonine-X-tyrosine activation motif usually found in enzymes of this family (Dorin *et al*., 1999).

The phylogenetic analysis has shown that PfMAPs do not specifically cluster with typical MAP kinases (Ward *et al*., 2004). By analysis of the *P. falciparum* kinome, Pfmap-2 was shown to be in a basal position relative to the MAPK family, which indicates no preferential relations to any of its subfamilies. Pfmap-1, on the contrary, is clearly associated with ERK8 that is not a part of typical three-component (MEKK-MEK-ERK) modules (Ward *et al*., 2004). This can indicate that other protozoan organisms, although use MAPK homologues, also do not have typical MEKK-MEK-ERK modules.

Many aspects of MAPK signaling in microbial organisms have yet remained unclear. In most cases, unknown are the precise mechanisms of regulation of the signal transduction pathways including the MAP kinase cascades; also not revealed is the PTK role in their activation. The examples presented in this review demonstrate differences, rather than similarity, of trigger mechanisms of the MAP kinase cascade activation in the higher and lower eukaryotes. Although the presence in signal protein networks in the series of unicellular eukaryotes of the growth receptor-like proteins, protein tyrosine kinases, Ras proteins, and Src homology 2 (SH2) of domain-containing proteins suggests that in lower eukaryotes, classical mechanisms of MAP kinase activation with participation of tyrosine phosphorylation also can take place, not all studied eukaryotic microorganisms have been revealed to contain the complete MEKK-MEK-MAPK cascades. In spite of this, it is evident that the MAP kinases activated by extracellular stimuli participate in regulation of fundamental cell processes: mating, proliferation, differentiation, and co-ordination of polarized growth, many of them being common to the higher and lower eukaryote cells. It is to be emphasized that in unicellular eukaryotes the signaling events that do not regulate MAPK activation, like probably in the higher eukaryote cells, may mediate aspects of tyrosine kinase signaling, such as ligand-stimulated cell survival, cytoskeletal rearrangements, cell migration, and chemotaxis (Ridley *et al*., 1992; Ridley and Hall, 1992).

Stress-activated protein kinases in the lower eukaryotes

The stress-activated protein kinases SAPK (JNK-p46, SAPK-p54, and p38-MAPK) revealed in the higher eukaryotes, related to ERK1/ERK2, are included in the MAP kinase family (Boulton *et al*., 1991; Derijard *et al*., 1994; Han *et al*., 1994; Kyriakis *et al*., 1994 Minden *et al*., 1994). Their activity also depends on processes of tyrosine and threonine phosphorylation; however, whereas the mammalian ERK1/2 are strictly activated by growth factors or phorbol esters (Kyriakis, 1994), SAPK show a high resistance to mitogens, but are activated in response to the heat and osmotic stress, ultraviolet irradiation, and action of cytokines (Brenner *et al*., 1989; Derijard *et al*., 1994; Han *et al*., 1994; Kyriakis *et al*., 1994; Cho *et al*., 2001). The initial chain of stress-activated SAPK pathway

is non-receptor tyrosine kinase, while the terminal chain is the protein product of oncogen *c-jun* (Fig. 3) (Davis, 1994; Derijard *et al*., 1994; Kyriakis *et al*., 1994; Hill and Treisman, 1995).

Stress-activated protein kinases (SAPKs) are also found in several unicellular eukaryotes. One of such kinases is in particular the HOG1 kinase in *Saccharomyces cerevisiae*, which is activated in response to osmotic stress (Brewster *et al*., 1993). Analysis of HOG1 sequences has revealed that an area near the NH_2-terminus contains a conservative sequence of about 300 amino acids, which has a high degree of homology with sequences in the kinase domain of MAP kinases: human ERK1 and *S. cerevisiae* FUS3. Like other MAP kinases, HOG1 has sites of phosphorylation at threonine 174 and at tyrosine 176 in the kinase domain VIII. HOG1 is phosphorylated at tyrosine in response to an increase of osmotic gradient. The HOG1 is the central kinase of the multi-cascade stress-activated signal pathway. This pathway is called the HOG pathway, as it controls the high osmolarity glycerol response, and is activated by a yet unidentified osmosensor or SH3-containing osmosensor protein Sho1p (Fig. 3) (Maeda *et al*., 1995; Posas and Saito, 1997; Raitt *et al*., 2000). The Sho1p coupled with protein STE50 that is a subunit of STE11, the MEKK family member, and together with STE20 PAK-like kinase activates STE11 by a yet unknown manner. The activated STE11 then apparently activates the PBS2 MEK by phosphorylating it. The PBS2 translates signal to HOG1. Stimulation is rapid, and phosphorylation of HOG1 at both serine/threonine and tyrosine can be observed 1 min after upshock (Maeda *et al*., 1995). The activated HOG1 is translocated to the nucleus (Reiser *et al*., 1999), where it is involved in stimulation of expression of about 150 genes (Posas *et al*., 2000). Several transcription factors including Hot1, Sko1 and Msp2/Msp4 are associated with the HOG pathway (Rep *et al*., 2000; Pascual-Ahuir *et al*., 2001).

Whereas nuclear targets of proteins participating in signal transduction via the HOG1 pathway are described sufficiently well, their cytoplasmic substrates in yeasts remain poorly studied. Mechanisms of regulatory action of signal proteins on effectors also need deciphering.

The kinase similar to the mammalian JNK/SAPK was found in *Toxoplasma gondii* (Roisin *et al*., 2000); properties of the *T. gondii* p53-JNK-like kinase are described in detail in the previous Section. Under experimental conditions this kinase together with other TgMAPKs was activated by Ca^{2+} influx to result in cell membrane depolarization. It is possible that under natural conditions the JNK-like kinase of *T. gondii* can be activated by interaction of tachyzoites with host cell. The precise mechanisms at present is not clear.

In the ciliate *Tetrahymena thermophila* the stress-activated MAP kinase MRK has been revealed; it is composed of 430 amino acid residues and resembles by its structure the human and yeast MAP kinases. Analysis of the primary structure has shown this kinase to have 40% of homology with the human ERK1 (Boulton *et al*., 1990) and 38%, with yeast HOG1 (Brewster *et al*., 1993) and the human p38 (Han *et al*., 1994), as well as a significant degree of homology with human stress-activated kinases (JNK1 and SAPK4). Use of phylogenetic analysis has allowed establishing that MRK of *Tetrahymena* are closer to p38 JNK/SAPK than to ERK. This kinase is activated *in vivo* after transfer of the cells cultivated at optimal temperature (35˚C) into conditions of physiological stress induced by a low temperature (15˚C) or an increase of the medium osmolarity. However, in MRK, unlike typical MAP kinases, the highly conservative site of phosphorylation at tyrosine in the kinase subdomain VIII is replaced by histidine (Thr226-Gly-His228). The recombinant MRK expressed in *Escherichia coli* phosphorylates a specific substrate (the MBP) and is autophosphorylated. When Thr 226 in the mutant recombinant protein is replaced by Ala, kinase loses the capability for autophosphorylation, which indicates an important role of this amino acid in providing MRK with function of dual specificity. Analysis of kinase biochemical properties has shown the mRNA transcript to increase significantly with decrease of temperature from 35˚ to 15˚C, the decrease rate being 0.8˚C/min. The highest mRNA expression of the MRK kinase mRNA is observed 1 hr after the decrease of temperature to 15˚C; this expression level exceeding about threefold the level of expression of MRK mRNA in intact cells is observed for the next 2 hr. The osmotic shock induced by sorbitol (100–200 mM) or NaCl (25–100 mM) also produces expression of MRK mRNA in *T. thermophila*. That the osmotic shock-induced MRK kinase belongs to the MAP kinase family was established by analysis of the immune kinase complex with use of the specific MAP kinase substrate (Nakashima *et al*., 1999).

The MRK-like kinase with identical biochemical characteristics and with the 90% structural homology of the primary structure was revealed in *Tetrahymena pyriformis* (Nakashima *et al*., 1999).

The structural-functional organization of the majority of signal pathways of unicellular microorganisms, in which stress-activated MAP kinases take part, has remained unclear so far. There are still too few works evaluating role of tyrosine phosphorylation in regulation of the stress-activated signal transduction pathways and in programming of processes aimed at maintenance of life activity of microbial cells under stress conditions (Schuller *et al*, 1994; Martinez-Pastor *et al*., 1996; Moskvina *et al*., 1998).

It is possible that, like in many metazoan cell systems, the up-stream regulation of the stress-activated signal pathway is independent of tyrosine phosphorylation. However, it is apparent that phosphorylation of microbial PTK at tyrosine is necessary to realize the enzyme kinase activity and to provide transduction of the stress signal into genome.

Participation of STAT-protein of *Dictyostelium* in intracellular signaling

At the end of the twentieth century, in mammalian cells the JAK/STAT pathways were revealed; they participated in the transcriptional activation of many cytokine- and growth factor-inducible genes. This illustrates how combinatorial interactions at the receptor itself can determine the kind of activated transcription complex and consequently the

nature of the target DNA sequence (Darnell *et al*., 1994; Ihle *et al*., 1994). STAT proteins that prior to receptor activation appear to be cytoplasmic (Schinndler *et al*., 1992) bind to their cognate receptor during activation (Fu and Zhang, 1993; Lutticken *et al*., 1994). Phosphorylation at conserved tyrosine in their COOH-terminus allows STAT dimerization; then an unknown mechanism allows them to leave the receptor and to migrate to the nucleus (Hill and Treisman, 1995). The STAT proteins, apart from the regions of Src homology (SH2 and SH3), contain the DNA-binding domain. In vertebrates and *Drosophila* the JAK-STAT pathway regulates cell proliferation, differentiation, adaptation, and hyperactivation and leads to tumor formation (Gatsios *et al*., 1998; Bode *et al*., 1999; Carballo *et al*., 1999).

The existence of the STAT pathways in the slime mold *Dictyostelium discoideum* (Kawata *et al*., 1997; Araki *et al*., 2003) and in the protozoan parasite *Entamoeba histolytica* (Cruz-Vera *et al*., 2003) has turned out to be unexpected. By the present time, the best studied are the STAT pathways of *D. discoideum*.

Among cytoplasmic components of these pathways found in the higher eukaryotes, three isoforms of this protein were identified in cells of *D. discoideum*: Dd-STATa, Dd-STATb and Dd-STATc, tyrosine kinases, and a number of regulatory proteins (see below) (Araki *et al*., 1998, 2003). The fungal STAT forms perform two different biological functions: Dd-STATa and DdSTATb are responsible for transduction of signals about cell differentiation (Kawata *et al*., 1997; Araki *et al*., 1998), whereas Dd-STATc regulates transduction of stress signals into genome (Araki *et al*., 2003).

Mechanisms of activation and pathways of signal transduction through STAT pathways in *D. discoideum* are different. Transduction of morphogenetical signals in slime mold cells is provided by the ligand-mediated activation of the serpentine receptor cAR1. The ligand for cAR1 is extracellular cAMP. This interaction results in producing autocrine factor DIF, a chlorinated phenyl alkanone (Berks *et al*., 1991) and activation of cytoplasmic PTK (Schweiger *et al*., 1990; Adler *et al*., 1996; Kim *et al*., 1999) that phosphorylates Dd-STATa at tyrosine, which leads to its activation: the capability for transport into the cell nucleus and binding with regulatory elements of target genes. The regulatory Dd-STATa action on processes of *D. discoideum* cell differentiation is realized through activation of transcription of genes *ecmA* and *ecmB* responsible for prestalk cell differentiation. It has been established that Dd-STATa can binds to both the *ecmA* activator and *ecmB* repressor elements in promoters of these genes, as both types of the regulators contain repeats of TTGA sequences, which are recognized by Dd-STATa (Kawata, 1996; Ceccarelli *et al*., 2000). Besides, Dd-STATa has recently been shown to act as a transcriptional activator of the gene *cudA*, whose expression is essential for correction of terminal differentiation of *D. discoideum* (Fukuzawa and Williams, 2000).

So far it is not yet clear whether the STATa pathway participates in regulation of *D. discoideum* apoptosis that is induced in spore cells located in the anterior part of the fruiting body or this process is controlled by other signal pathways. At any rate, such possibility can easily be admitted if to take into account that specificity of the STAT-mediated signal is determined in most cases by the type of the cells (Chin *et al*., 1997; Schindler, 1998).

Comparison of the primary structure and the biochemical analysis showed Dd-STATa to contain all main protein domains of the STAT group (Kawata, 1997), although the presence of the SH2-domain in the Dd-STATa molecule has turned out to be quite unexpected, as previously it was believed that this domain was unique for cell STAT proteins of multicellular organisms (Darnell, 1997).

The recombinant Dd-STATa is composed of approximately 700 amino acid residues and contains in its molecule C-terminal half the large areas similar by their primary structures with homologous areas of STAT proteins of the higher eukaryotes. For instance, Dd-STATa in the 241–262 sequences contains leucine-enriched repeats essential for phosphorylation of STAT1 (Improta *et al*., 1994). In the DNA-binding domain located in the N-terminal half of the protein, Dd-STATa has homology with human STAT5b and *Drosophila* D-STAT (Hou *et al*., 1996, Yan *et al*., 1996; Ehret *et al*., 2001) and contains homologous sequences concentrated around the conservative pair of valine residues that are essential for DNA binding in the STAT3 molecule (Horvath *et al*., 1995). Interestingly, the less conservative SH3-domain has been not detected in Dd-STAT, whereas the highly conservative SH2-domain revealed in all STAT proteins of multicellular organisms is present in both Dd-STAT isoforms.

The STATc pathway in *D. discoideum* is of great significance in maintaining viability of organisms under their unfavorable environmental conditions. So far, little is known about how the stress signal is transmitted through this pathway in microorganisms. The initial stages of this pathway and the exact mechanism of the Dd-STATc activation have not yet been established. Probably, this mechanism in *D. discoideum*, like in the majority of metazoa, is based on its abilities associated with JAK-like tyrosine kinase. Phosphorylation of Dd-STATc leads to its activation and subsequent translocation into the nucleus. So far it is absolutely unclear how this process is realized in the *D. discoideum* cells. It was only shown that Dd-STATc produced transcription of two genes: *gapA* and *rtoA* (Araki *et al*., 2003). The *gapA* gene – encoded protein of the same name represents a RasGAP-like protein necessary for normal *D. discoideum* cytokinesis (Adachi *et al*., 1997; Lee *et al*., 1997; Sakurai *et al*., 2001). GAPA has homology of its primary structure with mammalian IQGAP proteins and, like its homologues from mammalian cells, is effector for Rac and Cdc42 proteins participating in regulation of the actin cytoskeleton (Kuroda *et al*., 1996; Epp and Chant, 1997; Osman and Cerione, 1998). The RtoA protein encoded by the gene *rtoA* participates in regulation of the cell cycle (Wood *et al*., 1996). Both proteins play the key role in recovery of cells after a stress action and thereby participate in the cell systemic response to stress. Future studies using microorganisms are to be continued to provide insights into the roles and regulation of these proteins and their signaling pathways.

Conclusions

It is now well established that many intracellular processes are regulated by phosphorylation events. A particularly important role in regulation of such fundamental processes as growth, differentiation, and development is played by processes of tyrosine phosphorylation. This mechanism of regulation of signal transduction pathways was discovered in mammalian cells in the late 1980s and was long considered unique and evolutionarily associated with transition of organisms to multicellularity. However, in a number of unicellular eukaryotes (protozoa, yeasts, myxomycetes), tyrosine kinases activated by growth factors capable for autophosphorylation as well as Ras proteins, MAP kinases including the stress-activated ones, STAT proteins, etc. were identified. This argued in favor of the existence of a similar mechanism of signal transduction also in the lower eukaryotes. Thus, taking into account the above-exposed data, less and less doubts remain that the PTK signaling originates from primitive forms of microbial signal transduction, whose basis consists in processes of phosphorylation at tyrosine.

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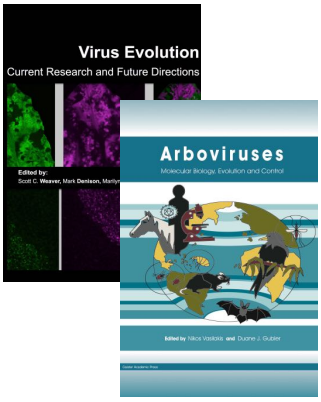
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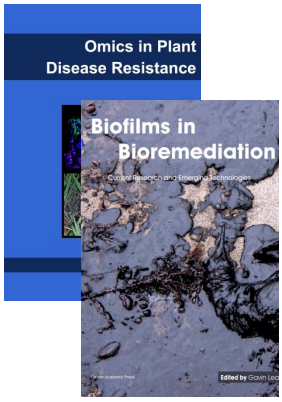
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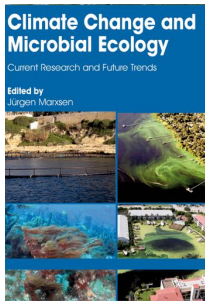
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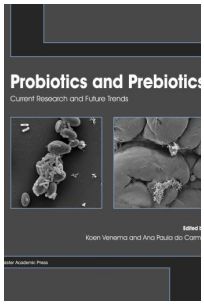
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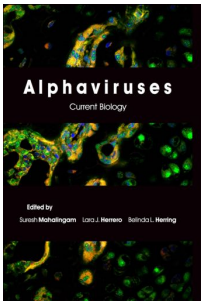
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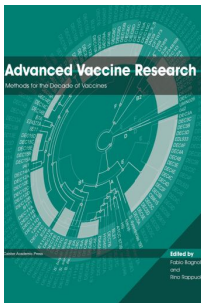


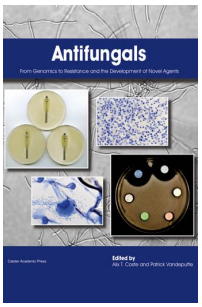












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