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Inhibition of MAPK Pathway by a Synthetic Peptide Corresponding to the Activation Segment of MAPK

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Mitogen-activated protein kinase (MAPK) is activated by phosphorylation within its activation segment. Upon phosphorylation, the activation segment refolds to provide the active conformation of the enzyme. We reported previously that a phosphorylation-sensitive secondary structure could be formed in a 26-amino-acid long synthetic peptide corresponding to the activation segment of *Xenopus* MAPK, termed IDA (Inter-DFG-APE) MAPK peptide (Tokmakov, A. A., *et al.* 1997, *Biochem. Biophys. Res. Commun.* 236, 243–247). Here, we show that unphosphorylated IDA MAPK peptide can inhibit *in vitro* both MAPK and MAPK kinase activities with the inhibition constants of 82 and 18 μM, respectively. Phosphorylated forms of the peptide were of little effect. IDA MAPK peptide did not inhibit significantly the activity of some other protein kinases, including MAPK homologue p38 kinase, suggesting the specificity for MAPK and MAPK kinase. Microinjection of unphosphorylated IDA MAPK peptide into immature *Xenopus* oocytes significantly suppressed progesterone-induced oocyte maturation by inhibiting activation of both MAPK and maturation promoting factor. Similar inhibition of maturation was registered upon oocyte treatment with another specific inhibitor of MAPK pathway, PD098059. These results depict IDA MAPK peptide as a selective inhibitor of the MAPK pathway that can be used for the investigations of MAPK-mediated signaling.

MAPK signal transduction pathway has been established to play a pivotal role in mediating cell responses to a variety of extracellular stimulus, such as growth factors, hormones, cytokines *etc.* (1). The involvement of MAPK in the processes of cell proliferation, differentiation, carcinogenesis and apoptosis is well documented (2). Many different intracellular transduction pathways converge to MAPK, making it a key regulatory enzyme and an important target for the directed action of regulatory agents. In mammalian cells, several MAPK enzymes have been identified that are regulated by distinct extracellular signals and have different substrate specificity, including ERKs (3, 4), JNK/SAPK (5, 6), and p38/Mpk2/RK (7, 8). ERKs are largely involved in regulating cellular responses to growth factors and proliferation, whereas JNK/SAPK and p38/Mpk2/RK are referred to as stress-activated protein kinases.

Several methods have been developed to block the activation of MAPK by extracellular stimuli, in order to investigate the involvement of MAPK pathway in the corresponding signal transduction. They include gene targeting and antisense oligonucleotide approaches, overexpression of dominant negative forms of Raf, MEK and MAPK itself, the use of MAPK-specific phosphatases and cell permeable inhibitors (9). Recently, highly specific synthetic inhibitor of MEK, PD098059, that selectively blocks the activation of ERKs of MAPK family (10, 11), and pyridinyl imidazole inhibitor of p38/Mpk/RK, called SB203580 (12), have been described. However, the molecular basis for their specificity is not quite clear.

All the enzymes of MAPK family are activated by a double phosphorylation on Tyr and Thr with the highly specific upstream MAPK kinases, that can only recognize the native conformation of MAPK (13, 14, 15). A partial activation of MAPK upon single site intramolecular autophosphorylation on Tyr also takes place (16, 17). The phosphorylation occurs within the activation segment residing in subdomains VII and VIII of the kinase domain, defined as the region between conserved DFG and APE motifs (18, 19, Fig. 1). Upon phosphorylation, conformational changes occur in the phosphorylation loop of the activation segment bringing the phosphorylated Tyr and Thr into alignment with the neighboring phosphate binding sites, thereby

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Abbreviations used: MAPK, mitogen-activated protein kinase; GST-MAPK, glutathione *S*-transferase-fusion protein of MAPK; IDA, inter-DFG-APE; MBP, myelin basic protein; MPF, maturation promoting factor; GVBD, germinal vesicle breakdown.

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providing domain rotation and the activation of the enzyme (20).

In the previous study, we have demonstrated that a 26-amino acid long synthetic peptide corresponding to the activation segment of *Xenopus* MAPK, termed IDA (Inter-DFG-APE) MAPK peptide, and the activation segment of native MAPK are virtually indistinguishable in terms of antibody recognition (21). The results suggested that IDA MAPK peptide might interfere with MAPK-mediated signaling by mimicking the activation segment of the native enzyme in biological interactions. In the present study, we show that unphosphorylated but not phosphorylated IDA MAPK peptide can inhibit *in vitro* both MAPK and MAPK kinase activities. The action of IDA MAPK peptide is rather specific, because some other protein kinases tested, including MAPK homologue p38 kinase, were not affected significantly. We used IDA MAPK peptide to inhibit the activation of MAPK pathway in *Xenopus* oocytes upon maturation and found that unphosphorylated and, to a less extent, phosphorylated peptide suppressed markedly both MAPK activation and oocyte maturation in response to progesterone. The activation of maturation promoting factor (MPF) was also inhibited in the microinjected cells. Similarly, another specific inhibitor of MAPK pathway, PD098059, suppressed MAPK and MPF activation, as well as progesterone-induced oocyte maturation. These results introduce IDA MAPK peptide as a selective inhibitor of MAPK pathway that might be used for signal transduction studies.

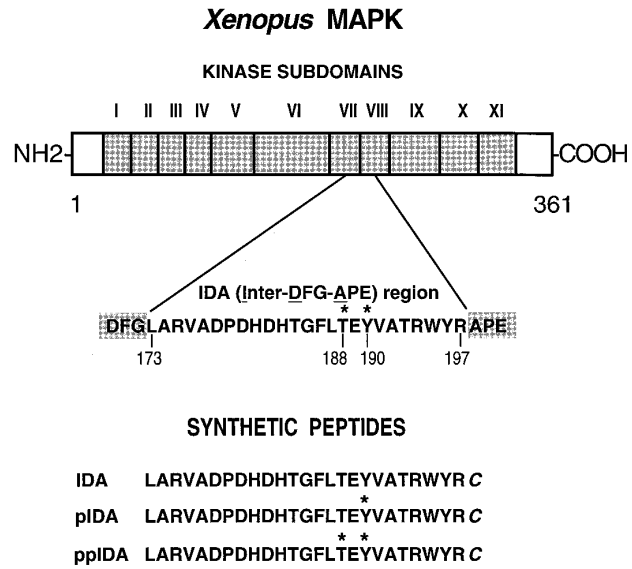

FIG. 1. Subdomain structure and amino acid sequence of IDA region of *Xenopus* MAP kinase and synthetic IDA MAPK peptides. Major subdomains of conserved protein kinase core including amino- and carboxyl-terminal extensions (361 amino acid residues in total) of *Xenopus* MAPK are shown. Amino acid sequences of IDA region and synthetic IDA MAPK peptides are also shown. Sites of regulatory phosphorylation in IDA region (Thr188 and Tyr190) and corresponding phosphorylated residues in IDA MAPK peptides are indicated by asterisks. The carboxyl-terminal cysteines shown in italics were introduced for the convenience of immunization.

MATERIALS AND METHODS

Materials. Pregnant mare serum gonadotropin (PMSG) was purchased from Biogenesis and progesterone was from Sigma. Collagenase (280 units/mg) was from Wako Pure Chemicals (Osaka). Glutathione-Sepharose 4B was obtained from Pharmacia. [γ-^{32}P]ATP (35020) and [^{125}I]protein A (68038) were from ICN, and poly(vinylidene difluoride) membranes were from Millipore. Anti-IDA MAPK antibody was raised against a synthetic IDA MAPK peptide (residues 173-197 of *Xenopus* MAPK) according to the method described previously (22). Recombinant GST-p38 kinase (506122) and PHAS-1 (516675) were purchased from Calbiochem. Myelin basic protein (MBP) and histone H1 were from Sigma. A specific inhibitor of the MAPK kinase activation, PD 098059, was purchased from Research Biochemicals International (Natick). Other chemicals of analytical grade were from Wako or Nakalai (Kyoto).

Enzyme preparations. Xenopus wild-type recombinant GST-MAPK (GST-MAPK WT) and kinase-negative mutant of MAPK (GST-MAPK KN) were expressed and purified in a form of glutathione S-transferase-fusion proteins, as described previously (23). *Xenopus* MAPK kinase was partially purified from the eggs of gonadotropin-injected frogs (1000 U/animal), according to the described method (24). Recombinant His6-MAPK kinase was kindly provided by Dr. Nishida (Kyoto University). c-Src from bovine brain and *Xenopus* tyrosine kinase (Xyk) from *Xenopus* oocytes were purified and assayed as described previously (25). EGFR was purified from A431 cells and assayed by autophosphorylation (26). Catalytic subunit of protein kinase A from bovine heart was prepared and assayed as described (27).

Protein kinase assays. In vitro protein kinase assays of recombinant GST-MAPK, His6-MAPK kinase, and purified MAPK kinase were carried out as described previously (23). MAPK and MPF activities in oocyte extracts were assessed by specific phosphate incorporation into MBP and histone H1, correspondingly. Groups of oocytes (7-10 cells) were homogenized in four volumes of 20 mM Tris-HCl buffer, pH 7.5, containing 60 mM β-glycerophosphate, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml leupeptin, 1% aprotonin, and 0.1 mM (p-amidinophenyl)-methane-sulfonyl fluoride hydrochloride. Extracts were obtained by centrifugation first at 10,000 I g for 5 min and then at 300,000 I g for 30 min. 2-5 μl of extract was used per 25 μl kinase assay under the conditions described previously for MAPK (28) and histone H1 kinase (29).

Immunoblotting. Samples of oocyte extracts (10 μg of total protein per lane) were subjected to immunoblot analysis using 200-fold diluted anti-IDA MAPK serum as described previously (23). The phosphorylation state of MAPK in the extracts was judged by the mobility shift of specific band.

Peptide preparations. Unphosphorylated and Tyr-phosphorylated IDA MAPK peptides of a sequence presented in Fig. 1, with the amino acid numbering corresponding to the residue positions in the original sequence of *Xenopus* MAPK (30), were synthesized and purified as described previously (21). Doubly phosphorylated IDA MAPK peptide was purchased from Sawady Technology Co (Tokyo). Amino acid sequences were verified by an Applied Biosystems Protein Sequencer (model 492). The identity and phosphorylation state of synthesized peptides were also confirmed by mass spectrometry using PerSeptive Biosystems Voyager Biospectrometry Workstation. A synthetic peptide, termed Cdc2 peptide, corresponding to residues 7-26 of the fission yeast cdc2 gene product was prepared as described (22).

Isolation of Xenopus oocytes. Female frogs were primed by dorsal lymph sac injection of PMSG (50 units per animal) 2-3 days prior to ovariectomy. Ovaries were surgically removed and treated with 0.5 mg/ml collagenase in OR2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM Hepes, pH 7.8) for 2 h at 21°C. After extensive wash in OR2 medium, stage VI immature oocytes were manually selected. Maturation was induced by the addition of progesterone to a final concentration of 10 μM. The occurrence of germinal vesicle breakdown (GVBD) was judged by the appearance of a white spot at the animal hemisphere of oocytes. Oocytes were microinjected with 50 nl of 5 mM IDA MAPK peptides dissolved in water using Drummond Nanoject automatic injector immediately after the addition of progesterone. It was confirmed that microinjections with water had no effect on the maturation. PD098059 was added at the beginning of progesterone treatment to a final concentration of 50 μM.

RESULTS AND DISCUSSION

The effect of IDA MAPK peptide on the activity of MAPK kinase was tested in a reaction mixture containing activated MAPK kinase purified from *Xenopus* eggs and recombinant catalytically inactive GST-MAPK KN (Fig. 2A). The phosphorylation of GST-MAPK KN was greatly inhibited in the presence of unphosphorylated peptide. The inhibition constant (Ki) determined for unphosphorylated peptide in a detailed kinetic analysis was 18 μM. Tyr-phosphorylated IDA MAPK peptide was inhibitory at higher concentrations, while doubly phosphorylated peptide had no effect on MAPK kinase activity up to 500 μM concentration. In contrast to the phosphorylation of exogenous substrate, only partial inhibition of recombinant His6-MAPK kinase autophosphorylation with the unphosphorylated IDA MAPK peptide was detected in the same range of the peptide concentration (Table 1). The lowered inhibitory potential of the phosphorylated forms of IDA MAPK peptide in this assay is consistent with the poor recognition of activated (phosphorylated) MAPK by MAPK kinase.

We next investigated the effect of IDA MAPK peptide on the activity of MAPK *in vitro.* Phosphorylation of an exogenous substrate, MBP, (Fig. 2B, lower band) but not autophosphorylation of recombinant *Xenopus* MAPK WT (Fig. 2B, upper band) was found to be inhibited in the presence of unphosphorylated IDA MAPK peptide. In a separate experiment, the inhibition constant was estimated to be 82 μM. Phosphorylated forms of the peptide exerted no inhibitory effect on MAPK activity up to 500 μM.

We have also investigated the effect of IDA MAPK peptide on the activation of MAPK by MAPK kinase (Fig. 2C). The enzymatic system contained activated purified MAPK kinase from *Xenopus* eggs, recombinant catalytically active MAPK WT and MBP. As anticipated, unphosphorylated IDA MAPK peptide inhibited both MAPK kinase- and MAPK-mediated reactions of this assay, i.e. MBP phosphorylation and MAPK kinase-dependent MAPK phosphorylation (Fig. 2C). Correspondingly, activation of MAPK with MAPK kinase was blocked. The resulting IC_{50} for MBP phosphorylation in this assay was below 100 μM (data not shown). Phosphorylated IDA MAPK peptide exerted only minor inhibitory effect on the activation of MAPK with MAPK kinase in the assay system employed (Fig. 2C). We also confirmed the inhibition of MBP phosphorylation by IDA MAPK peptide in *Xenopus* egg extracts (data not shown).

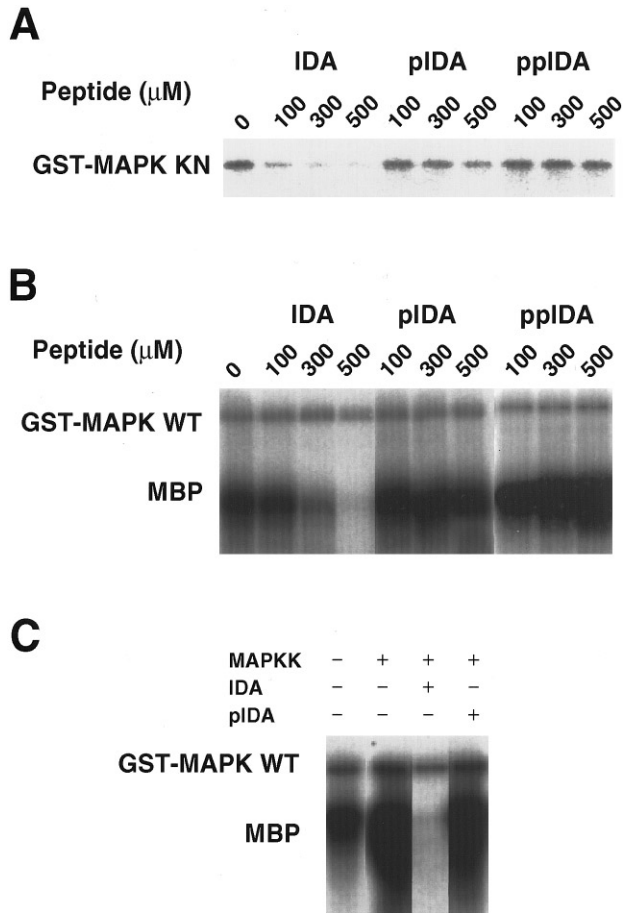

FIG. 2. Inhibitory effect of IDA MAPK peptide on MAPK kinase and MAPK activity *in vitro.* The effect of unphosphorylated and phosphorylated IDA MAPK peptide on the phosphorylation of recombinant kinase-negative MAPK (GST-MAPK KN) with MAPK kinase purified from *Xenopus* eggs (*A*) and phosphorylation of MBP with recombinant wild-type MAPK (GST-MAPK WT) accompanied by its autophosphorylation (*B*) is presented. In panel (*C*), unphosphorylated or phosphorylated IDA MAPK peptide (500 μM) was added to reaction mixture containing purified MAPK kinase and recombinant wild-type MAPK, with MBP as a substrate.

From the results presented in Fig. 2, we concluded that IDA MAPK peptide can inhibit phosphorylation and activation of MAPK by MAPK kinase, as well as the activity of MAPK towards exogenous substrate. However, autophosphorylation of MAPK could not be inhibited by IDA MAPK peptide. Strictly intramolecular character of *Xenopus* MAPK autophosphorylation

TABLE 1

Effect of IDA MAPK Peptide on the Activity of Protein Kinases *in Vitro*

Protein kinase	Substrate	Activity (% control)
Xenopus MAPK	auto[a]	100
	MBP	9
Xenopus MAPK kinase	auto	67
	MAPK	4
Protein kinase A	histone H1	89
c-Src	auto	115
	Cdc2 peptide	98
Xyk	auto	117
	Cdc2 peptide	91
EGFR	auto	35
p38 kinase	auto	97
	PHAS-1	125

Note. The protein kinases were assayed in the presence or absence of 200 μM unphosphorylated IDA MAPK peptide as described under Materials and Methods. Activities are given relative to controls without the peptide. Values are the mean from two separate experiments.

[a] Autophosphorylation of the kinase.

(23) may account for the lack of the inhibition. In the inactive state, the substrate-binding site of MAPK is blocked intramolecularly by autoinhibitory IDA region. It prevents MAPK interaction with IDA MAPK peptide and inhibition of autophosphorylation. In the active state, both IDA MAPK peptide and exogenous substrate gain an access to the unblocked substrate-binding site of MAPK, resulting in the competitive inhibition of the enzyme activity by the peptide. In this case, IDA MAPK peptide might probably take the position occupied by the unphosphorylated autoinhibitory IDA region in inactive MAPK, whereas phosphorylated forms of the peptide can not be readily accommodated in the same position because of the structural limitations imposed by phosphorylation. We reported previously, that the difference between the secondary structures of phosphorylated and unphosphorylated activation segment could be detected on the peptide level (21). The competitive character of MAPK inhibition by IDA MAPK peptide was confirmed in a separate experiment (data not shown).

It has been demonstrated previously, that the short peptides corresponding to consensus sequence for peptide substrate recognition site by MAPK may serve as competitive inhibitors of the enzyme (31). Also, the synthetic peptides corresponding to sequences surrounding the sites phosphorylated with ERK *in vivo* have been shown to selectively inhibit MAPK in the millimolar range of concentrations (32). In these cases, the peptides could be phosphorylated by MAPK. In comparison, IDA MAPK peptide was found to be a poor substrate of both MAPK and MAPK kinase, as reported earlier for the shorter peptides containing MAPK phosphorylation sites (14, 33). At rather high concentrations (1 mM), it could be weakly phosphorylated on Thr by both kinases independently of its Tyr-phosphorylation (data not shown). Nevertheless, IDA MAPK peptide displayed more than one order higher affinity for MAPK than the above-mentioned peptide substrates and pseudosubstrates. Interestingly, shorter nonapeptides derived from IDA MAPK sequence including TEY motif of regulatory phosphorylation failed to inhibit the activity of MAPK up to 0.5 mM (data not shown).

As expected, IDA MAPK peptide could not inhibit significantly the activity of some other protein kinases such as protein kinase A, c-Src, Xyk, EGFR (Table 1). However, the peptide was found to be phosphorylated on Tyr by EGFR (data not shown) and partially inhibited EGFR autophosphorylation. Importantly, the peptide didn't affect the activity of MAPK homologue p38 kinase (Table 1), further suggesting the specificity for MAPK and MAPK kinase.

The results of *in vitro* experiments suggested that IDA MAPK peptide may also serve as an inhibitor of MAPK pathway *in vivo*. To address this question we investigated the effect of microinjected IDA MAPK peptide on the MAPK activation and progesterone-induced maturation of *Xenopus* oocytes. Recently, the importance of MAPK pathway in oocyte maturation has been established (34). MAPK is activated during *Xenopus* oocyte maturation concomitantly with MPF (a complex of $p34^{cdc2}$ kinase and cyclin B) followed by GVBD and chromosome condensation. Microinjection of constitutively active thiophosphorylated MAPK into immature oocytes could activate MPF and promote GVBD without any hormonal stimulation (35). On the other hand, injection of MAPK specific phosphatase CL100 or a neutralizing antibody against MAPK kinase inhibited or delayed progesterone-induced oocyte maturation and MAPK activation (29, 36). As shown in Fig. 3A, oocyte maturation in response to progesterone was markedly suppressed by the injection of unphosphorylated IDA MAPK peptide, however, doubly phosphorylated peptide could also inhibit maturation to a less extent (Fig. 3A). The injections of IDA MAPK peptide at 5 mM, but not 0.5 mM were found to be effective (data not shown). The calculations show, that assuming the average volume of an oocyte equal to about 1 μl and isotropic distribution of the injected material, the injection of 50 nl of 5 mM peptide would result in 250 μM intracellular peptide concentration, which surpasses the inhibition constant values determined for *in vitro* enzymatic assays. Therefore, the injection of 5 mM IDA MAPK makes possible the shutdown of MAPK pathway in oocytes. Importantly, the treatment of oocytes with another specific inhibitor of MAPK pathway, PD098059, that blocks the activity of MAPK kinase, suppressed maturation to the same extent as the injections of IDA MAPK peptide (Fig. 3A).

We further measured the activity of MAPK and MPF in non-treated, IDA MAPK peptide-microinjected, and

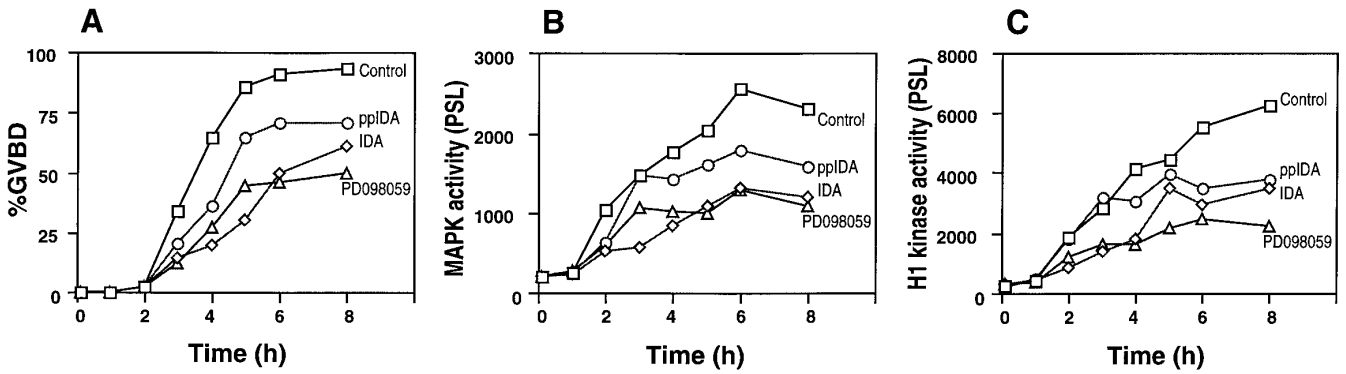

FIG. 3. Suppression of progesterone-induced oocyte maturation by microinjection of IDA MAPK peptide. *Xenopus* oocytes were injected with 50 nl of 5 mM unphosphorylated or doubly phosphorylated IDA MAPK peptides (a total of about a hundred oocytes for each peptide), or PD098059 compound (50 μM) was added to the cells at the beginning of progesterone treatment. In panel (A), time course of maturation, as judged by GVBD, is shown. Time courses of MAPK and histone H1 kinase activation in the process of maturation are shown in panels (*B*) and (*C*), respectively. The phosphorylation of MBP and H1 was quantified by a Bioimaging analyzer (BAS2000, FUJI Film) in arbitrary units (PSL).

PD098059-treated *Xenopus* oocytes in the course of maturation. In the control non-treated oocytes, both H1 kinase (a measure of MPF activity) and MAPK were greatly (more than 20 times) activated after 6-8 hours of progesterone treatment (Fig. 3B, C). By this time, almost all MAPK is found in its phosphorylated active form, without the change in the total intracellular content, as judged by immunoblotting (data not shown). Significantly lower activation of MAPK and MPF took place in the oocytes microinjected with the unphosphorylated peptide or oocytes treated with PD098059 (Fig. 3B, C). In this case, considerable fraction of MAPK remained unphosphorylated by the end of progesterone treatment, as confirmed by immunoblotting (data not shown). Microinjection of phosphorylated IDA peptide also inhibited the activation of MAPK and MPF, however, the effect was less prominent than in the case when unphosphorylated peptide was injected (Fig. 3).

High specificity of both IDA MAPK peptide and PD098059 suggests that they suppress oocyte maturation by inhibiting MAPK activation. It should be noted that both inhibitors of MAPK pathway failed to prevent completely the activation of MAPK and MPF, as well as progesterone-induced maturation of oocytes. Only partial inhibition of *Xenopus* oocyte maturation with PD098059 has been reported recently in a similar experiment (37). It is possible that intracellular concentration of the inhibitors was not sufficient to effectively shut down MAPK pathway in oocytes. However, estimated intracellular concentration of microinjected IDA MAPK peptide (250 μM, see above) exceeded several times the values of inhibition constants measured *in vitro*. Also, the concentration of PD098059 used (50 μM) has been demonstrated to completely block the activity of MAPK pathway in several cell types (10, 38, 39). On the other hand, PD098059 failed to inhibit completely the *in vivo* activation of MAPK in Swiss 3T3 cells stimulated with high concentrations of agonists that are powerful activators of MAPK kinase and Raf (11). We suggest, therefore, that a little residual activation of MAPK kinase induced by progesterone in the oocytes treated with MAPK kinase inhibitors might bring about, in the long run, the registered essential activation of MAPK.

Although phosphorylated IDA MAPK peptide failed to inhibit the activity of either MAPK or MAPK kinase *in vitro* (Fig. 2), it suppressed MAPK activation, when microinjected in progesterone-treated oocytes (Fig. 3). It is possible that microinjected phosphorylated peptide is subjected to dephosphorylation *in ovo* upon several hours long exposure to intracellular phosphatases during maturation. Then, it can specifically inhibit MAPK pathway in its dephosphorylated form. Also, the phosphorylated peptide might affect the activity of some other enzymes, related to protein phosphorylation, for instance, phosphatases.

Alltogether, the results of the present study demonstrate that IDA MAPK peptide can serve as a specific inhibitor of MAPK transduction pathway and might be useful for the investigations of the physiological role of this pathway in different biological systems. The fact that IDA MAPK peptide is derived from an autoinhibitory fragment of MAPK, that is recognized and modified by the upstream MAPK kinase, may account for the unique bifunctional mode of the peptide action: it can inhibit both MAPK and MAPK kinase with the relatively good inhibition constants. The inhibition involves catalytically active forms of the enzymes (Fig. 2) and seems to be reversible and competitive for both enzymes (data not shown), as it could be expected from the known mode of MAPK activation. As shorter nonapeptides derived from IDA MAPK sequence failed to inhibit MAPK activity (data not shown), the length of IDA MAPK peptide seems to be related to the increased affinity of its interactions. Conformational

flexibility and ability of IDA MAPK peptide to folding, as demonstrated earlier (21), may also add to its inhibitory potential. Moreover, it may account for the specificity of the peptide action: protein kinases of different families differ in the sequence of IDA region and, even within the same kinase family, folding of IDA region may be different. For example, published crystal structures of ERK2 and p38 kinase revealed that the conformation of the activation segment is different in these MAP kinases (40, 41). It might explain the specific inhibition of *Xenopus* MAPK, highly homologous to mammalian ERK2, but not p38 kinase by IDA MAPK peptide (Table 1).

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