Communication

The Dual Specificity Phosphatases M3/6 and MKP-3 Are Highly Selective for Inactivation of Distinct Mitogen-activated Protein Kinases*

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The mitogen-activated protein (MAP) kinase family includes extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38/RK/CSBP (p38) as structurally and functionally distinct enzyme classes. Here we describe two new dual specificity phosphatases of the CL100/MKP-1 family that are selective for inactivating ERK or JNK/SAPK and p38 MAP kinases when expressed in COS-7 cells. M3/6 is the first phosphatase of this family to display highly specific inactivation of JNK/SAPK and p38 MAP kinases. Although stress-induced activation of p54 SAPKβ, p46 SAPKγ (JNK1) or p38 MAP kinases is abolished upon co-transfection with increasing amounts of M3/6 plasmid, epidermal growth factor-stimulated ERK1 is remarkably insensitive even to the highest levels of M3/6 expression obtained. In contrast to M3/6, the dual specificity phosphatase MKP-3 is selective for inactivation of ERK family MAP kinases. Low level expression of MKP-3 blocks totally epidermal growth factor-stimulated ERK1, whereas stress-induced activation of p54 SAPKβ and p38 MAP kinases is inhibited only partially under identical conditions. Selective regulation by M3/6 and MKP-3 was also observed upon chronic MAP kinase activation by constitutive p21ras GTPases. Hence, although M3/6 expression effectively blocked p54 SAPKβ activation by p21ras (G12V), ERK1 activated by p21ras (G12V) was insensitive to this phosphatase. ERK1 activation by oncogenic p21ras was, however, blocked totally by co-expression of MKP-3. This is the first report demonstrating reciprocally selective inhibition of different MAP kinases by two distinct dual specificity phosphatases.

The mitogen activated protein (MAP) kinase family comprises the extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38/RK/CSBP (p38) as three structurally and functionally distinct enzyme classes (1–7). ERK family members are activated by a variety of growth and differentiation factors, while MAP kinases of the JNK/SAPK, and p38 class are activated preferentially by cellular stresses, and inflammatory cytokines (1–5, 7–12). Activated MAP kinases phosphorylate a range of cellular substrates, including additional kinases and several transcription factors (7, 13–16). MAP kinase-dependent regulation of diverse targets indicates a critical role orchestrating many varied and important cellular processes. Likely functions include a pivotal role for ERK in mediating neuronal differentiation in PC12 cells as well as growth factor-stimulated proliferation and oncogenic transformation in fibroblasts (17–21). Recent investigations also support the view that activation of JNK/SAPK and p38 MAP kinases are critical in processes mediating platelet aggregation and secretion, in generation of inflammatory cytokines as well as in triggering of apoptotic death in a range of cell types (6, 12, 22–25).

Full activation of MAP kinases requires dual phosphorylation on threonine and tyrosine residues within kinase domain VIII. Although several upstream kinases acting selectively on ERK, JNK/SAPK, or p38 family members have now been identified (14, 16, 26), dephosphorylation by specific phosphatases may also play a critical role. An emerging class of dual specificity phosphatases inactivate MAP kinases through dephosphorylating both threonine and tyrosine residues critical for enzymatic activation (27). To date, 10 distinct dual specificity phosphatase gene family members have been identified. These include CL100/MKP-1 (identical to 3CH134) (28–31), VHR (32), PAC1 (33, 34), hVH-2 (also cloned as MKP-2 and TYP-1) (35–37), hVH-3 (also known as B23) (38, 39), MKP-3 (identical to rVH-6 and orthologue of PYS1) (40–42), MKP-X (orthologue of PYS1) (40, 42), MKP-4,2 (43), and M3/6 (44). These phosphatases all posses a characteristic extended active site motif VXVHXXGXXSRTXXXXYX(L/I)YM (where X is any amino acid) and NH2-terminal CH2 domains possessing homology to the cell cycle regulator Cdc25 phosphatase (45). As part of an investigation into the biological function of dual specificity phosphatases, we have observed highly specific regulation of MAP kinases by two recently cloned family members, M3/6 and MKP-3 (40, 44), and we describe these observations in this report.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s cell culture medium was obtained from Life Technologies, Inc., protein A-Sepharose4 fast flow was from Pharmacia Biotech Inc., murine EGF was from Promega (Madison, WI), and [γ-32P]ATP (5000 Ci/mmol) was from DuPont de Nemours International S. A. (Regensdorf, Switzerland). Anti-RA monoclonal antibodies 12CA5 or HA.11 were purchased from Boehringer Mannheim (Rotkreuz, Switzerland) or Ruwag Diagnostics (Zurich, Switzerland), respectively. Anti-Myc monoclonal antibody 9E10 was from Dr. Glaser (Basel), protein A/G-horseradish peroxidase conjugate from Pierce (Zurich, Switzerland), whereas horseradish peroxidase conjugates of EGF, epidermal growth factor.

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† The abbreviations used are: MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activated protein kinase; HA, hemagglutinin; EGF, epidermal growth factor.
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RESULTS AND DISCUSSION

To establish a system to allow assessment of MAP kinase regulation by M3/6 and MKP-3, HA-tagged ERK1, p54 SAPKβ, and p38 MAP kinase were expressed in COS-7 cells and activated by exposure to a number of acute stimuli. Although ERK1 is stimulated by EGF, H2O2, and menadione (vitamin K), p54 SAPKβ and p38 are activated following cellular exposure to anisomycin, sodium arsenite, H2O2, UV light, and sorbitol. p38 MAP kinase but not p54 SAPKβ also undergoes activation by menadione (Fig. 1). EGF (ERK1), UV (p54 SAPKβ), and H2O2 (p38) were selected as stimuli to test inhibitory regulation by dual specificity phosphatases.

M3/6 has been expressed previously in COS cells and shown to be ineffective as an inhibitor of serum-stimulated ERK2 phosphorylation (44). In accordance with these observations, although increasing the concentration of Myc-M3/6 plasmid in transfections from 0.1 to 2.0 μg resulted in a dose-dependent increase in immunodetectable Myc-tagged protein (not shown), EGF-dependent enzymatic activation of ERK1 was inhibited less than 30% even at the maximum levels of M3/6 obtained (Fig. 2). Interestingly, however, parallel experiments demonstrated clearly that M3/6 blocks stress-induced activation of both p54 SAPKβ and p38 MAP kinases with maximal inhibition observed when cells were transfected using only 0.5–1.0 μg of plasmid (Fig. 2). Identical inhibition by M3/6 was observed when p54 SAPKβ and p38 MAP kinases were activated by UV, anisomycin, or H2O2. Also, p46 HA-SAPKγ (JNK1) activated by anisomycin is inhibited identically over the range of M3/6 expression levels obtained in COS-7 cells (not shown). Together, these observations demonstrate that M3/6 displays highly selective inhibition of JNK/SAPK and p38 MAP kinases when expressed in mammalian cells.

We have reported previously that MKP-3 expressed in COS-7 cells blocks totally both endogenous and heterologously expressed ERK2 activation following stimulation with growth factors (40). In a more detailed analysis we have now co-transfected ERK1 with a range of Myc-M3/6 plasmid levels (0.1–2.0 μg), which results in a dose-dependent increase in immunodetectable protein (not shown). This approach demonstrates that MKP-3 also blocks EGF-stimulated ERK1 activation and that this inhibition is maximal when cells are transfected with...
0.5–1.0 μg of MKP-3 plasmid (Fig. 3). Basal ERK1 activity is also abolished when cells are transfected with 1.0 μg or more of MKP-3 plasmid (Fig. 3). In contrast to observations with ERK1 and ERK2, stress-activated p54 SAPKβ or p38 MAP kinases were suppressed only partially using 0.5–1.0 μg of plasmid with near complete blockade observed only when cells were co-transfected with 2.0 μg of MKP-3 plasmid (Fig. 3). These observations demonstrate that MKP-3 appears highly selective for inactivation of the ERK family of MAP kinases.

It is now clear that several members of the p21^{ras} superfamily of GTPases are linked to the activation of different MAP kinase family members. For instance, constitutively active p21^{ras} (G12V) stimulates activation of ERK (8), and this may underlie mitogenesis and cellular transformation by this oncoprotein (19, 46). In addition, recent studies have shown that mutationally activated versions of the p21^{ras} GTPase family members p21^{ras-} and p21^{ras+} elicit enzymatic activation of both JNK/SAPK and p38 but not ERK MAP kinases (10, 11). Interestingly, p21^{ras-} and p21^{ras+} also stimulate DNA synthesis and appear to play a critical role in mediating mitogenesis and transformation by oncogenic p21^{ras} (47, 48). To test whether M3/6 or MKP-3 retain their activity and selectivity for inhibiting MAP kinases undergoing oncogenic activation, COS-7 cells were triple transfected with constitutively active p21^{ras} (G12V) or p21^{ras-} (G12V) together with different MAP kinases and increasing concentrations of plasmid for MKP-3 or M3/6. This experiment shows clearly that as with acute stimulation, M3/6 inhibited effectively p54 SAPKβ activation by constitutive p21^{ras} (G12V) (Fig. 4). Also, as observed with short term exposure to growth factor, MKP-3 blocked ERK1 activation by oncogenic p21^{ras} (G12V) (Fig. 4), whereas M3/6 was completely ineffective (not shown). Importantly, co-transfection with increasing concentration of either MKP-3 or M3/6 plasmid does not alter the level of immunodetectable p54 HA-SAPKβ or HA-ERK1 (Fig. 4). The ability of MKP-3 to suppress chronic MAP kinase activation by p21^{ras} (G12V) together with its clear selectivity for ERK family members could indicate a physiological role as an inhibitor of proliferation or even a tumor suppressor.

This is the first account of two dual specificity phosphatases, M3/6 and MKP-3, displaying reciprocal selectivity for inactivating ERK or JNK/SAPK and p38 MAP kinases. The remarkable inactivity of M3/6 against ERK family members indicates a high degree of specificity between MAP kinase family members and has not been demonstrated previously for any phosphatase of this class. These observations with M3/6 and MKP-3 are distinct from experiments using the dual specificity phosphatases PAC1, MKP-2, and CL100/MKP-1, which appear moderately selective when expressed in mammalian cells (49). Our data on selective enzymatic inhibition indicate that regul-
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labeled expression of MKP-3 and M3/6 could be a critical parameter in both short and long term control of cell function by different MAP kinases. MKP-3 and M3/6 are unique amongst dual specificity phosphatases insofar that they are both localized in cytosolic compartments (40, 44), whereas other members of this gene family are nuclear (33, 35, 37, 38). The M3/6 gene also possesses a translated complex trinucleotide repeat resulting in multiple serine and glycine residues within the COOH-terminal third of the protein (44). Whether either of these novel characteristics underly their selectivity for MAP kinase inactivation is currently under investigation in our laboratories.

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