A High-throughput Assay for Phosphoprotein-specific Phosphatase Activity in Cellular Extracts*

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Protein phosphatases undo the post-translational modifications of kinase-signaling networks, but phosphatase activation in cells is difficult to measure and interpret. Here, we report the design of a quantitative and high-throughput assay platform for monitoring cellular phosphatase activity toward specific phosphoprotein targets. Protein substrates of interest are purified recombinantly, phosphorylated in vitro using the upstream kinase, and adsorbed to 96-well plates. Total phosphatase extracts from cells are then added to trigger a solid-phase dephosphorylation reaction. After stopping the reaction, phosphoprotein levels are quantified by ELISA with a phospho-specific antibody, and the loss of phospho-specific immunoreactivity is used as the readout of phosphatase activity. We illustrate the generality of the method by developing specific phosphatase-activity assays for the three canonical mitogen-activated protein phospho-kinases: ERK, JNK, and p38. The assays capture changes in activity with a dynamic range of 25–100-fold and are sensitive to a limit of detection below 25,000 cells. When applied to cytokine-induced signaling, the assays revealed complex and dynamic regulation of phosphatases suggesting cross-communication and a means for cellular memory. Our assay platform should be beneficial for phosphoproteomic surveys and computational-systems models of signaling, where phosphatases are known to be important but their activities are rarely measured. Molecular & Cellular Proteomics 12: 10.1074/mcp.O112.024059, 797–806, 2013.

Phosphatases (PPases)1 reset post-translational modifications by kinases and thus help to sculpt the phosphoproteome (1–3). Once thought of as global attenuators of phosphorylations by kinases and thus help to sculpt the phosphoproteome, PPases are now known to recognize specific subsets of phosphoprotein targets (4–7). Cellular PPase activity toward these phosphoprotein targets is regulated at multiple levels. PPases can be induced transcriptionally (8–10), for example, and their catalytic efficiency is further controlled by diverse post-translational modifications (11–15). Notably, misregulation of PPases has been implicated in various inherited disorders (16, 17) and in diseases such as cancer (18, 19).

Multiple computational studies have indicated that PPases are especially important for the system-level properties of a signaling network (20–23). However, mathematically encoding explicit PPase species is problematic, because many PPases act on multiple substrates (2, 3), and each phosphosite can often be dephosphorylated by multiple PPases (24, 25). Consequently, PPases are often modeled as generic species that are tonically active, although some models include transcriptional regulation in an effort to capture feedback control (21, 23, 26–28). The unfortunate result of this simplification is a model whose generic PPases cannot be constrained by experimental observations. Thus, for network modeling of phosphorylation cascades, there is a need for measurement platforms that capture total PPase activity toward key signaling transducers.

The activity of purified PPases is readily measured with artificial colorimetric substrates (29) or chromogenic indicators of released inorganic phosphate (30, 31). Yet, neither of these detection strategies is compatible with total cellular extracts. Improved selectivity can be achieved with fluorescently labeled peptide substrates (32, 33), but these peptides still lack the structural requirements important for specific recognition by PPases (4–7). One can work around the promiscuity of such substrates by gel electrophoresis of crude extracts and then enzyme renaturation (34, 35), although this focuses on the PPases rather than the phosphosubstrates. Perhaps the clearest way to measure specific PPase activity is with the phosphosubstrate itself. However, previous assays have used radiolabeled substrates that are short-lived and must be precipitated away from the released 32P signal (36, 37), which reduces throughput. More recently, nonradioactive ELISA formats have been explored using broad phospho-motif antibodies (38), but the crossreactivity of such antibodies precludes their use for monitoring specific dephosphorylation events on key signaling proteins. Despite many

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1 The abbreviations used are: PPase, phosphatase; MAPK, mitogen activated protein kinase; ERK, extracellular regulated kinase; JNK, c-Jun N-terminal kinase; TXY, Thr-X-Tyr; DUSP, dual-specificity PPase; GST, glutathione S-transferase; EGF, epidermal growth factor; TNF, tumor necrosis factor; 4PL, four-parameter logistic; CHX, cycloheximide.
Assay for Phosphoprotein-specific Phosphatase Activity

decades of research on PPases, an assay has not been developed that is quantitative, high-throughput, sensitive, and specific for the conversion of phosphosubstrates.

Here, we report the general design of such an assay and its proof-of-principle application to the PPases deactivating the three canonical mitogen-activated protein kinases (MAPKs): extracellular-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38. MAPK pathways are critical signal-transduction modules that control proliferation, death-survival, differentiation, and stress responses throughout eukaryotes (39, 40). MAPKs are all regulated by phosphorylation of a Thr-X-Tyr (TXY) motif in their activation loop, which is catalyzed by dual-specificity MAPK kinases (MAP2Ks). Complete TXY dephosphorylation is catalyzed by dual-specificity PPases (DUSPs) called MAPK PPases (MKPs) (3, 7). The TXY motif can also be deactivated by the joint action of serine-threonine PPases and tyrosine PPases (41–44). For our assay development and validation, bisphosphorylated MAPKs provide a prototypical phosphosubstrate under complex negative regulation that changes dynamically in response to environmental stimuli (8–14). However, the format described here should generalize to any phosphoprotein that can be prepared in vitro and can be monitored with a high-quality phosphospecific antibody.

**EXPERIMENTAL PROCEDURES**

*Plasmid Construction*—Recombinant MAPKs and their upstream constitutively active MAP2Ks were cloned by PCR into pGEX-4T-1 glutathione S-transferase (GST) fusion plasmids containing triple epitope tags for Flag or HA. Rat ERK2 (Addgene, Cambridge, MA; plasmid #8974) (45) was cloned into the BamHI and SalI sites of pGEX-4T-1 (3×Flag) by PCR with the primers gcgcggatccatggcggcg-gccggccgcccggg (forward) and gcgcgtgctactagaagctgtctgtgctgtg (reverse) followed by digestion with BamHI and Sall. Human JNK1 (Addgene plasmid #13798) (46) was cloned into the BamHI and Sall sites of pGEX-4T-1 (3×Flag) by PCR with the primers gcgcggccacctgagcagcatgt cgtatctgtcggtc (reverse) followed by digestion with BamHI and Sall. Human MKK7a1-EE (Addgene plasmid #14540) (50) was cloned into the BamHI and SalI sites of pGEX-4T-1 (3×HA) by PCR with the primers gcgcggccacctgagcagcatgtcgtatctgtcggtc (forward) followed by digestion with BamHI and SalI. Murine MEK1-DD (Addgene plasmid #15268) (48) was cloned into the BamHI and SalI sites of pGEX-4T-1 (3×Flag) by PCR with the primers gcgcggccacctgagcagcatgtcgtatctgtcggtc (forward) followed by digestion with BamHI and SalI. Human MKK4-EE (Addgene plasmid #14813) (49) was cloned into the BamHI and Sall sites of pGEX-4T-1 (3×Flag) by PCR with the primers gcgcggccacctgagcagcatgtcgtatctgtcggtc (forward) followed by digestion with BamHI and SalI. Human MKK6-EE (Addgene plasmid #13518) (51) was cloned into the BamHI and Sall sites of pGEX-4T-1 (3×HA) by PCR with the primers gcgcagatctatgtctcagtc-gaaaagcag (forward) and gcgcgctgctactagtctccaagtaaatcttac (reverse) followed by digestion with BglII and SalI. The ligation products were transformed into electrocompetent E. coli (DH10B; Invitrogen, Grand Island, NY) and ampicillin-resistant clones were screened by endonuclease digestion and sequencing.

**Protein Induction and Purification**—Sequence-verified clones were transformed into low-copy E. coli (C41 DE3; Avidis, Saint-Beauzire, France) and grown at 37 °C until an optical density of 0.6–0.8 was achieved. The GST-fusion proteins were induced with isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C under the following conditions: GST-3xFlag-ERK2—2 mM IPTG for 5 h, GST-3xFlag-JNK1—0.4 mM IPTG for 4.5 h, GST-3xFlag-p38α—0.4 mM IPTG for 4 h, GST-3xHA-MEK1-DD—2 mM IPTG for 3 h, GST-3xHA-MKK4-EE—0.4 mM IPTG for 5 h, GST-3xHA-MKK7-EE—1 mM IPTG for 5 h, and GST-3xHA-MKK6-EE—0.4 mM IPTG for 4.5 h. Cells were harvested by centrifugation, resuspended in 7.5 ml TNE lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μg/ml pepstatin) per 250 ml cell culture, and lysed with lysozyme and deoxycholate. Bacterial extracts were clarified by centrifugation and incubated overnight at 4 °C with glutathione-coated agarose beads (Sigma, St. Louis, MO). The bead-bound proteins were washed three times with ice-cold PBS + 0.5% Triton X-100 and then twice with PBS. MAP2Ks were eluted from the beads with 10 mM glutathione in 50 mM Tris pH 8.0 and concentrated with ultrafiltration columns (Millipore, Billerica, MA) before phosphorylation of MAPKs.

**In Vitro Phosphorylation of MAPKs**—Phosphorylation of the bead-bound MAPKs was accomplished by incubating the beads with their corresponding, concentrated MAP2Ks diluted in kinase assay buffer (10 mM Tris pH 7.5, 1 mM ATP, 15 mM MgCl2, 2.5 mM beta-glycerophosphate, 0.5 mM Na3VO4, 0.5 mM EDTA, 0.2 mM DTT) for 24 h at 37 °C. The phosphorylation reactions were halted by the addition of excess EDTA and the beads were washed three times with PBS. The phosphorylated MAPKs were purified by thrombin digest and quantified by SDS-PAGE with Coomassie staining.

**In Vitro Dephosphorylation of pMAPKs**—To verify phosphospecificity of the ELISAs, 0.5–1 μg of pMAPK was dephosphorylated with 1250 units lambda PPase (New England Biolabs, Ipswich, MA) in 50 mM HEPES (pH 7.5), 0.1% 3-(3-cholamidopropyl)dimethylammonio)-propanesulfonate, 100 mM NaCl, 1 mM MnCl2 for 2 h at 30 °C.

**Cell Culture, Lysis, and Stimulation**—HT-29 cells (ATCC, Manassas, VA) were cultured according to the manufacturer’s specifications. For the cytokine time courses, HT-29 cells were seeded at 50,000 cells/cm2, grown for 24 h, then treated with 200 U/ml interferon gamma (IFNγ) for an additional 24 h (52). IFNγ-sensitized cells were then treated with 100 ng/ml epidermal growth factor (EGF), 100 ng/ml tumor necrosis factor (TNF), or both for the indicated times. Cells were washed once with ice-cold PBS and then lysed in a PPase lysis buffer (50 mM HEPES (pH 7.5), 0.05% saponin, 50 mM 2-mercaptoethanol (βME), 1 mM dithiothreitol (DTT), 2 mM MgCl2, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 μg/ml pepstatin). Whole cell lysates were incubated on ice for 15 min then clarified by centrifugation at 16,000 x g for 15 min at 4 °C. Clarified lysates were frozen as single-use aliquots at −80 °C.

For the EGF prestimulation experiment, cells were seeded and sensitized as described above, then treated with 100 ng/ml EGF for 2 h followed by 100 ng/ml TNF for 15 min. Cells were washed once with ice-cold PBS then lysed in radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 μg/ml pepstatin). Whole cell lysates were incubated, clarified, and stored as described above.

**High-throughput PPase Assay**—High-protein-binding polystyrene microtiter plates (Corning Costar, Lowell, MA) were coated overnight with the indicated amounts of recombinant phosphorylated MAPK.
The plates were then washed three times with PBS + 0.1% Tween-20 (PBS-T), and the dephosphorylation reaction was initiated by the addition of cell lysates (~27,000 cells/well for pERK2 and pp38 and ~7,000 cells/well for pJNK). The dephosphorylation reaction was allowed to proceed at 30 °C for the indicated times, and the reaction was terminated by the addition of a concentrated PPase-inhibitor mixture (20 mM NaPP, 60 mM NaF, 400 μM Na3VO4). The plates were then washed three times with PPase-inhibitor wash solution (10 mM NaPP, 30 mM NaF, 200 μM Na3VO4 diluted in PBS-T) before proceeding.

**Enzyme-linked Immunosorbent Assay (ELISA)—**Inhibitor-washed plates were blocked for 1 h at room temperature with blocking buffer (PBS-T + 5% BSA) before incubation with one of the following primary antibodies diluted in blocking buffer: anti-phospho-p44/p42 (Cell Signaling Technology, Danvers, MA; 1:1000, 1 h), anti-phospho-p38 (Cell Signaling Technology; 1:1000, 1 h), or anti-phospho-SAPK/JNK (Cell Signaling Technology; 1:50, 3 h). The plates were then washed three times with PBS-T and incubated with blocking buffer containing biotinylated goat anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA; 1:10,000) for 1 h at room temperature. After three washes with PBS-T, the plates were incubated with streptavidin-HRP (R&D Systems, Minneapolis, MN; 1:200) for 30 min at room temperature. The plates were again washed three times with PBS-T and incubated with ELISA detection reagents (R&D Systems) for 5–15 min. The ELISA reaction was halted by the addition of 1 M H2SO4, and the signals were measured by spectrophotometry at 450 nm with plate background correction at 540 nm. PPase activation was monitored by the decrease in phosphorylation level of the lysate-treated wells relative to untreated control wells. Relative activity values were calculated by four-parameter logistic regression using a serial dilution of concentrated PPase lysate.

**Western Blotting—**PPase or RIPA lysates were resolved on 10% or 12% polyacrylamide gels and transferred to 0.45 μm polyvinylidene fluoride (Millipore, Billerica, MA). Membranes were blocked with 0.5 Odyssey blocking buffer (Licor, Lincoln, NB) diluted in PBS and probed with chicken anti-tubulin (Abcam, Cambridge, MA) at 1:5000 dilution and the following antibodies at 1:1000 dilution: rabbit anti-phospho-SAPK/JNK (Cell Signaling Technology), anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phospho-p38, anti-IκBα, anti-MKP5 (Cell Signaling Technology), anti-DUSP16/MKP7 (Novus Biologicals, Littleton, CO). The membranes were then probed with 800 CW goat anti-rabbit (Licor) and 680 RD donkey anti-chicken (Licor) secondary antibodies at 1:20,000 dilution and visualized by fluorescence on a Licor Odyssey imager (Licor).

** Lentiviral shRNA Knockdown—**The following pLKO.1 shRNA constructs were obtained through the RNAi Consortium: shMKP5 (#1: TRCN0000001885 and #2: TRCN0000001887) and shMKP7 (#1: TRCN0000005203 and #2: TRCN0000005217) (Open Biosystems, Lafayette, CO). Lentiviruses were packaged, transduced into HT-29 cells, and selected with 2 μg/ml puromycin as previously described (53).

**Statistics—**Comparisons between means was performed by Student’s t test after correcting for error propagation. Comparisons between control and shMKP knockdown lines were performed by Fisher’s method of combining probabilities (54).

**RESULTS**

We developed the general assay in a solid-phase format with adsorbed recombinant phosphoprotein as a substrate (Fig. 1A). Phosphoprotein-coated plates are then incubated with cell extracts containing endogenous PPases. The dephosphorylation reaction proceeds for a fixed time before quenching and removal of the lysates. Loss of protein phosphorylation is quantified by an ELISA with high-quality phosphospecific antibodies and used as the measure of phosphoprotein-specific PPase activity (Fig. 1B). This format captures the activity of endogenous PPases toward specific phospho-substrates in a high-throughput and quantitative manner.

We chose to develop the assay for PPases targeting the three canonical MAPKs: ERK, JNK, and p38. These proteins are ideal substrates for the assay because their upstream MAP2Ks are known and can be rendered constitutively active with phosphomimetic substitutions in their activation loop. Furthermore, the modest size of the MAPKs allows them to be purified as GST fusion proteins in bacteria. Bead-bound MAPKs from bacterial extracts were phosphorylated with soluble active MAP2Ks, washed extensively, and then eluted by thrombin cleavage of the GST-MAPK linker. We observed no degradation of the MAPKs during the phosphorylation-elution steps (supplemental Fig. S1), and the phosphorylation stoichiometry was equal to or less than that observed when endogenous MAPKs were phosphorylated during cytokine stimulation (supplemental Fig. S2). Phospho-MAPK (pMAPK) yields ranged from ~50–150 μg, allowing for hundreds of microtiter wells to be coated from a 250 ml bacterial culture.

**Quantifying Post-translational Changes in Recombinant MAPKs by Phosphoprotein-specific ELISA—**We optimized phosphosubstrate adsorption by immobilizing twofold serial dilutions of pERK, pJNK, or pp38 on polystyrene plates and
buffer containing saponin (for gentle permeabilization of the membranes), disrupting their native activity. We optimized a PPase lysis assay—

The success of the solid-phase reaction was dependent on the amount of immobilized pMAPKs.

To investigate the phosphospecificity of the ELISA detection, immobilized pMAPKs were treated with recombinant lambda (λ) PPase. λ PPase is a broad-specificity enzyme with reactivity toward phosphorylated serine, threonine, and tyrosine residues. We found that λ PPase treatment of each pMAPK strongly reduced the measured ELISA signal (Fig. 3A–3C, upper panels). The reduction coincided with the loss of pMAPK immunoreactivity as determined by immunoblotting (Fig. 3A–3C, lower panels). In MAPK purifications that had not been phosphorylated by MAP2Ks, we observed some background phosphorylation (Fig. 4A–4C). λ PPase treatment samples were analyzed by immunoblotting for pERK (A), pJNK (B), or pp38 (C) with Flag or total MAPK used as a loading control. Data are shown as the mean ± S.E. of three independent assay replicates.

Next, it was important to determine whether the ELISA readout was strongly dependent on MAPK phosphorylation. To investigate the phosphospecificity of the ELISA detection, immobilized pMAPKs were treated with recombinant lambda (λ) PPase. λ PPase is a broad-specificity enzyme with reactivity toward phosphorylated serine, threonine, and tyrosine residues. We found that λ PPase treatment of each pMAPK strongly reduced the measured ELISA signal (Fig. 3A–3C, upper panels). The reduction coincided with the loss of pMAPK immunoreactivity as determined by immunoblotting (Fig. 3A–3C, lower panels). In MAPK purifications that had not been phosphorylated by MAP2Ks, we observed some background phosphorylation (Fig. 4A–4C). λ PPase treatment samples were analyzed by immunoblotting for pERK (A), pJNK (B), or pp38 (C) with Flag or total MAPK used as a loading control. Data are shown as the mean ± S.E. of three independent assay replicates.

To capture the dephosphorylation activity of cell extracts, we quantitatively mapped the assay readout to changes in the enzymatic properties of the sample. First, we optimized the duration of lysate incubation on the pMAPK-coated plate to ensure that the dephosphorylation reaction was proceeding at a constant reaction velocity. When extracts (~275,000 cells/well for pERK and pp38, and ~70,000 cells/well for pJNK) were compared with lysis buffer controls, we observed clear lysate-specific activity that increased linearly for at least 30 min (Fig. 4A–4C). PPase activity plateaued at longer incubation times, likely because of phosphosubstrate depletion. We also observed higher background losses of phosphorylation on pJNK-coated plates (Fig. 4B), consistent with the increased lability of the phosphoprotein in cell extracts (55). For plasma membrane, DTT and βME (to maintain stable reducing conditions and avoid inadvertent oxidation of PPase catalytic cysteines) and MgCl₂ (to provide Mg²⁺ cofactor that is critical for PPase catalysis). These conditions were stringent enough to extract cytosolic PPases while minimizing disruptions in PPase activity.

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The ELISA measurement of pMAPK levels is phosphospecific. A–C (upper panels), immobilized MAPKs or pMAPKs were incubated in the presence (+) or absence (−) of lambda (λ) PPase and the resulting phosphorylation levels of pERK (A), pJNK (B), or pp38 (C) were measured by ELISA. A–C (lower panels), matched λ PPase-treated samples were analyzed by immunoblotting for pERK (A), pJNK (B), or pp38 (C) with Flag or total MAPK used as a loading control. Data are shown as the mean ± S.E. of three independent assay replicates.

FIG. 2. ELISA-based quantification of recombinant pMAPKs adsorbed to 96-well plates. A–C, Twofold dilution series (relative level 1.0 = −400 ng) of pERK (A), pJNK (B), and pp38 (C) were adsorbed onto polystyrene microtiter plates and detected by ELISA using phospho-specific antibodies. The detection saturates at ~75 ng for pERK (A), ~400 ng for pJNK (B), and ~75 ng for pp38 (C). Arrows indicate the quantity of pMAPKs used in the PPase assay. Data are shown as the mean ± S.E. of three independent assay replicates.

FIG. 3. The ELISA measurement of pMAPK levels is phosphospecific. A–C (upper panels), immobilized MAPKs or pMAPKs were incubated in the presence (+) or absence (−) of lambda (λ) PPase and the resulting phosphorylation levels of pERK (A), pJNK (B), or pp38 (C) were measured by ELISA. A–C (lower panels), matched λ PPase-treated samples were analyzed by immunoblotting for pERK (A), pJNK (B), or pp38 (C) with Flag or total MAPK used as a loading control. Data are shown as the mean ± S.E. of three independent assay replicates.

FIG. 4. Early reaction kinetics of the MAPK PPase activity assays are linear with time. A–C, immobilized pERK (A), pJNK (B), or pp38 (C) were treated with saturating concentrations of PPase lysate (see Fig. 5) or lysis buffer for the indicated times. Arrows indicate the incubation times used in the optimized assays. Gray markers indicate time points where the kinetics have saturated. Data are shown as the mean ± S.E. of three independent assay replicates.
all substrates, we identified the linear regime of dephosphor-
ulation and used this incubation time in all subsequent exper-
iments (Fig. 4A–4C, arrows).

We next explored how measured PPase activity changed
with different amounts of cell extract. This titration was de-
signed to characterize the quantitative performance, sensitiv-
ty, and dynamic range of the assay. Compared with lysis
buffer controls, measured PPase activities increased sharply
before saturating with extracts above 50,000 cells/well
(for pERK and pp38) and 12,000 cells/well (for pJNK)
(Fig. 5A–5C). The response behavior was accurately captured
by a four-parameter logistic (4PL) model of the kind frequently
used for ELISA standard curves (58). The steep slopes of
activity combined with overall assay precision (2.1% for
pERK, 8.8% for pJNK, and 3.6% for pp38) indicate that the
absolute limits of detection (59) were less than 30,000 cells/
well. By focusing the assay conditions in the middle of the
response curve (Fig. 5A–5C, arrows), we were able to capture
5-10-fold down-regulation or up-regulation in activity. This
dynamic range is sufficient to capture the relative changes in
PPase activity reported in cells (60, 61).

Last, to ensure that the measured activities were indeed
catalyzed by PPases, we performed the assays in the pres-
ence of various PPase inhibitors. We used sodium pyrophos-
phate (NaPP) and sodium fluoride (NaF) to inhibit Ser-Thr
PPases and sodium orthovanadate (Na3VO4) to inhibit Tyr
PPases. Combined treatment with NaPP, NaF, and Na3VO4
abolished PPase activity in all assays (Fig. 6A–6C), whereas
individual inhibitors showed differential reductions in PPase
activity depending on the pMAPK. In general, NaF and NaPP
were more effective at reducing measured PPase activity
compared with Na3VO4. These findings agree with previous
reports indicating that the anti-pMAPK antibodies used in the
ELISAs react with monophosphorylated pTXY in addition to
bisphosphorylated pTXpY (56, 57). Furthermore, the results
suggest that MKPs, which are inhibited by Tyr PPase inhibi-
tors but not by Ser-Thr PPase inhibitors (8, 62, 63), are only
partly responsible for cellular pMAPK dephosphorylation ac-
tivity (see below). We were unable to test the effect of Tyr
PPase inhibition on pJNK PPase activity, because Na3VO4-
treated lysates created a false-positive pJNK signal (Fig. 6B
and supplemental Fig. S3). This false ELISA signal was not
dependent on the immobilized pJNK and was instead caused
by the anti-pJNK antibody and the PPase lysate (supplemen-
tal Fig. S3A, 3B). The false signal was not caused by the
hyperphosphorylation of endogenous JNK, because immuno-
blot analysis of inhibitor-treated PPase lysates showed no increase in pJNK levels in response to Na3VO4 (supplemental Fig. S3C). Nevertheless, combining Na3VO4 with Ser-Thr PPase inhibitors eliminated the artifact and returned the measured JNK PPase activity to background levels (Fig. 6B).

We conclude that the pMAPK PPase assays are accurate, reproducible, sensitive, and specific for endogenous PPase activity in cell extracts.

Application of the MAPK PPase Assays to Cytokine-induced Signaling—To validate the assay in a biological context, we measured the dynamics of PPase activity in cytokine-treated HT-29 colon adenocarcinoma cells (see Experimental Procedures). Cells were sensitized with IFN-γ and then stimulated with receptor-saturating concentrations of TNF, EGF, or both, and MAPK PPase activities were monitored for up to 8 h (Fig. 7A–7F). These conditions were chosen to coincide with a large systematic study of signaling in these cells, in which MAPK activities or phosphorylation were measured previously (52, 64–67).

We found that all MAPK-specific PPase activities changed in response to cytokine stimulation, but the regulation of each PPase activity was distinct. For example, EGF was consistently the most effective inducer of MAPK PPase activity (Fig. 7A–7C), in agreement with the widespread up-regulation of DUSPs/MKPs triggered by EGF (68). However, EGF caused a sustained activation of JNK PPase while eliciting only a transient activation of p38 PPase and a negligible activation of ERK PPase (Fig. 7D), suggesting pathway specificity in the response. We also observed clear non-additivity in PPase activation when TNF was added together with EGF. TNF costimulation reduced or eliminated MAPK PPase activation triggered by EGF (Fig. 7D–7E), providing an explanation for the higher EGF-induced ERK activity observed when combined with TNF (64). The PPase activity profiles are thus consistent with the literature and quantify the negative regulation of MAPKs in a manner that is more directly interpretable.

Last, we sought to test a prediction of our in vitro PPase assays in a cellular context. The strongest activation event we observed in our data set was for JNK PPases in cells stimulated with EGF for 2 h (eightfold activation, Fig. 7B). This result was surprising, because EGF is a weak activator of JNK signaling (64, 68, 69). We reasoned that the high PPase activity, if true, would create a form of cellular “memory” that would dampen the response of cells to future JNK-activating stimuli, such as TNF (69). To test this prediction, we pretreated cells with EGF for 2 h and then stimulated with TNF for 15 min (Fig. 8A), which maximally activates JNK in HT-29 cells.
EGF up-regulates MKP5 and MKP7 to prime cells against future JNK-activating stimuli. A, HT-29 cells were seeded at 50,000 cells/cm², sensitized with 200 U/ml IFN-γ, and stimulated with 100 ng/ml EGF for 2 h to upregulate JNK PPase activity, followed by 100 ng/ml TNF for 15 min. B, cells with or without 100 ng/ml EGF prestimulation or 100 ng/ml TNF stimulation were analyzed for pJNK, pp38, or total IκBα levels with JNK1 and tubulin used as loading controls. pIκBα appears as an upshifted band on the total IκBα immunoblot. C, replicated densitometry of the results shown in (B). D, inhibition of protein synthesis inhibits EGF-induced pJNK memory. Cells were pretreated with 20 μg/ml cycloheximide (CHX) before EGF–TNF stimulation as described in (A). E, replicated densitometry of the results shown in (D). F and G, EGF up-regulates MKP5 and MKP7. Cells were stimulated with 100 ng/ml EGF for 2 h and analyzed for MKP5 or MKP7 by immunoblotting with tubulin used as a loading control. Replicated densitometry is shown in the upper panels. H, I, shRNA-mediated knockdown of MKP5 or MKP7. Cells were transduced with lentiviruses, selected, and analyzed for MKP5 or MKP7, pJNK, or pp38 with tubulin used as a loading control. Relative densitometry for the extent of knockdown is shown beneath the MKP5 and MKP7 immunoblots. J, Knockdown of MKP5 or MKP7 reduces EGF-induced pJNK memory. shMKP5 or shMKP7 cells were stimulated with EGF–TNF as described in (A) and analyzed for pJNK by immunoblotting. Replicated densitometry is shown compared with shGFp control cells. Data are shown as the mean ± S.E. of 3–4 biological replicates. Single asterisk indicates p < 0.05, and double asterisk indicates p < 0.01.

(64). Early TNF-induced JNK signaling is a key point of signal integration with receptor tyrosine kinases and has been shown to be a strong predictor of TNF-induced apoptosis (65, 70). We found that EGF prestimulation led to a significant reduction in early TNF-induced pJNK compared with cells that had not been prestimulated (p < 0.01, Fig. 8B–8C). There was also a slight attenuation of TNF-induced pp38, consistent with the twofold increase in p38 PPase activity caused by EGF prestimulation for 2 h (Figs. 7C–7D, 8B). Conversely, we observed no change in TNF-induced IκBα phosphorylation and degradation, indicating that EGF does not globally upregulate PPase activity (Fig. 8B). These experiments suggested that the measured JNK PPase activity stimulated by EGF could be relevant for JNK activation in vivo.

EGF elicits waves of gene expression that feed back on earlier signaling events, and PPases are a major component of this transcriptional signature (68, 71). The gradual kinetics of JNK PPases (Fig. 7D) suggested that changes in activity could be because of EGF-stimulated gene expression. We tested this possibility by inhibiting protein synthesis with cycloheximide (CHX) and found that EGF-induced pJNK memory was significantly reduced in CHX-treated cells (p < 0.05, Fig. 8D–E). To identify specific PPases involved, we took a candidate approach and focused on cytoplasmic MKPs that target pJNK (3) and are regulated by EGF (68).

The JNK/p38-selective PPases, MKP5 and MKP7, were both significantly up-regulated after 2 h of EGF treatment (p < 0.05, Fig. 8F–8G), suggesting that they could contribute to overall JNK PPase activity. To determine whether MKP5 and MKP7 were specifically involved in EGF-induced memory, we stably knocked down each PPase with one of two independent shRNA targeting sequences (Fig. 8H–8I, upper). Despite partial knockdown, shMKP5 and shMKP7 cells showed variable increases in basal p38 phosphorylation, confirming biological efficacy (Fig. 8H–8I, upper). Conversely, there were negligible changes in basal JNK phosphorylation, likely because of minimal upstream pathway activity in the absence of cytokine stimulation. When shRNA-expressing cells were prestimulated with EGF and then challenged with TNF, we found that the paired shMKP5 and shMKP7 lines had reduced memory compared with the shGFp control (p < 0.01, Fig. 8J). Together, we conclude that EGF-induced memory is partly caused by the coordinate up-regulation of MKP5 and MKP7, leading to elevated JNK PPase activity (Fig. 7D). The results overall indicate that the activity assay platform accurately reflect facets of PPase regulation in vivo.
DISCUSSION

In this work, we develop a conceptually different approach for high-throughput monitoring of PPase activity in cell extracts. Rather than striving to measure specific PPase isoforms or holoenzymes, we inverted the assay design to focus on the collective PPase activity toward specific phosphosubstrates. This modification streamlines the assay by eliminating the need for affinity-capture reagents, much like the reverse-phase format that is now widely used for phosphoproteomics (72).

We focused here on PPases that act on the pMAPKs, because these phosphoproteins are negatively regulated at multiple levels and are known to contribute to network function (8–14). However, the overall assay format is general and should apply to many phosphoproteins of interest. The protein targets of most commercial phosphospecific antibodies have been cloned and their upstream kinases identified. In the future, our assay could be adapted to large panels of recombinant proteins that have been phosphorylated in vitro or, perhaps more easily, in vivo using bacitracin vectors (73). Encouragingly, in vivo preparations of phosphoprotein have shown large improvements in yield and ELISA immunoreactivity (supplemental Fig. S4), suggesting a way to streamline expansion of the assay. Thus far, the dephosphorylation reaction conditions for the three MAPK PPases are sufficiently comparable that groups of phosphoproteins could conceivably be combined in a spotted-array format for multiplex activity profiling (74).

With the PPase activity assays reported here, we envision that the most-immediate applications will be toward providing empirical constraints on systems models of MAPK signaling (21, 23, 26–28). Lumped “MAPK PPase” species in such models should conform to the quantitative and temporal changes measured with the assay, thereby reducing the danger of model overfitting. Alternatively, one could exploit the remarkable sensitivity of the assays for high-throughput screening. We routinely detect activity in extracts from ~25,000 cells, making the assay compatible with 96-well cell-based screens for activators or inhibitors of MAPK PPases. From intracellular networks to small-molecule libraries, we believe that our PPase assay format will prove to be a useful profiling tool for this understudied class of signaling enzymes.

Although simple and effective, the assay has its caveats. The passive adsorption of phosphosubstrate onto the microplate surface (Fig. 1A) could prevent access to docking sites that are important for proper PPase recognition (4, 75). Therefore, the physiological relevance of any measured activity changes should be followed up with independent approaches (Fig. 8). As a biochemical method, our assay also overlooks the role of subcellular localization, which is important for certain PPases (76, 77). In the future, the assay format could be further elaborated to provide additional data on PPase regulation in subcellular compartments. For example, our current PPase lysis buffer uses saponin to gently permeabilize cells and extract cytoplasmic proteins, but the cholesterol-poor inner nuclear envelope is largely unaffected (78). It may be possible to pellet the insoluble material from saponin lysates and extract with a nonionic detergent to isolate nuclear PPase activity in the same cell preparation.

More globally, we see no reason why the overall assay format cannot be adapted to a mass-spectrometry paradigm through triple SILAC labeling (79). Medium- and heavy-labeled arginine could be used to prepare identical phosphosubstrate pools from cells treated with irreversible PPase inhibitors, such as calyculin A (80) and pervanadate (81). After dialyzing away the unreacted inhibitor, one of the pools could be incubated with a PPase lysate as prepared here (containing only light arginine). The second pool would then be added at the end as an internal reference after the PPase reaction has been quenched, with activity read out by the loss of phosphate between the medium and heavy pools. Mass spectrometry has been used to define optimal substrates for specific PPases (82), but the inverted approach we propose has not been described to our knowledge.

Our follow-up experiments with EGF–induced pJNK memory illustrate how activity data obtained with the assay can be further pursued to hone in on specific PPase species. Interestingly, the observed memory is only partially dependent on up-regulated MKP5 and MKP7. This emphasizes the redundancy in the network and raises the possibility that there are other PPases, which may be post-translationally controlled and contribute to the residual memory in CHX-treated cells. The ~40% reduction in pJNK caused by EGF prestimulation may seem modest considering the ~eightfold increase in JNK PPase activity under the same conditions (Figs. 7D, 8B–8C). However, this behavior is qualitatively consistent with models of amplified enzymatic signaling cascades, where signal amplitude is much more sensitive to kinase rates than PPase rates (20). The rich information content of the JNK pathway (65, 70) suggests that PPase regulation may play a critical role in fine-tuning an appropriate signaling response.

The overall literature of kinase signaling exceeds that of PPase signaling by a factor of ~eight. Given the wealth of knowledge and available reagents for phosphoproteomics, it seems wise to leverage these resources for studying the negative regulators of protein phosphorylation. Our method lays the groundwork for such a strategy, which may help to unravel the time-dependent and context-specific control of the enzymatic “brakes” for signal-transduction networks (83).

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[This article contains supplemental Figs. S1 to S4.]
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