Proteomics Analysis of Protein Kinases by Target Class-selective Prefractionation and Tandem Mass Spectrometry*

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Protein kinases constitute a large superfamily of enzymes with key regulatory functions in nearly all signal transmis-
sion processes of eukaryotic cells. However, due to their relatively low abundance compared with the vast majority of cellular proteins, currently available proteomics tech-
niques do not permit the comprehensive biochemical characterization of protein kinases. To address these lim-
itations, we have developed a prefractionation strategy that uses a combination of immobilized low molecular weight inhibitors for the selective affinity capture of protein kinases. This approach resulted in the direct purifi-
cation of cell type-specific sets of expressed protein ki-
nases, and more than 140 different members of this enzyme family could be detected by LC-MS/MS. Furthermore the enrichment technique combined with phos-
phopeptide fractionation led to the identification of more than 200 different phosphorylation sites on protein ki-
nases, which often remain occluded in global phospho-
proteome analysis. As the phosphorylation states of pro-
tein kinases can provide a readout for the signaling activities within a cellular system, kinase-selective phos-
phoproteomics based on the procedures described here has the potential to become an important tool in signal transduction analysis. Molecular & Cellular Proteomics 6:537–547, 2007.

The basic principle of reversible phosphorylation of proteins represents a fundamental regulatory mechanism of central importance to signal transmission in higher organisms. In human cells, most protein phosphorylation events are catalyzed by the more than 500 members of the protein kinase superfamily of enzymes, also known as the human kinome (1). Therefore, protein kinases take the center stage in the complex signaling networks regulating a wide range of biological processes such as cell proliferation, differentiation, and survival (2, 3). Many members of the protein kinase superfamily are positively or negatively regulated by either interacting proteins or through specific phosphorylation events mediated by other protein kinases. For example, in the case of the central cell cycle regulator cyclin-dependent kinase 2 (CDK2),1 both phosphorylation in the kinase domain activa-
tion loop on Thr-160 by CDK activating kinase and binding of cyclin A or cyclin E are required for the full activation of CDK2 activity, whereas phosphorylation at Thr-14 and Tyr-15 by the kinases Wee1 or Myt1 or binding of the specific protein inhibitors p21CIP1 or p27KIP1 result in inhibition of catalytic activity (4). Upon transition to the active state, various protein kinases further undergo autophosphorylation reactions on specific serine, threonine, or tyrosine residues, which can stabilize their active conformations or lead to the generation of additional docking sites for phosphorylation-dependent protein-protein interactions (5, 6). Although only a fraction of all hu-
man protein kinases has been studied in mechanistic detail, extrapolation of available data suggests that autophosphorylation reactions and/or phosphorylation events by upstream kinases can provide a readout for the cellular activities of almost all members of the human protein kinase superfamily. However, because protein kinases are usually of much lower abundance than their cellular substrate proteins, previous global phosphoproteome studies have resulted in the identi-
fication of rather small numbers of kinase-derived phos-
phopeptides (7–10). Even the largest dataset of 2002 phos-
phorylation sites reported in a study by Gygi and co-workers (8) included only 61 from protein kinases. Consequently it appears that selective prefractionation strategies are required for the in-depth analysis of protein kinases and their cellular phosphorylation sites with currently available MS techniques.

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Received, November 15, 2006, and in revised form, November 30, 2006

Published, MCP Papers in Press, December 27, 2006, DOI 10.1074/mcp.T600062-MCP200

*The abbreviations used are: CDK, cyclin-dependent kinase; DMF, dimethylformamide; EDC, N-ethyl-N'-[3-dimethylaminopropyl]carbo-
diimide hydrochloride; bisX, bisindolylmaleimide X; purB, purvalanol B; AMP-PNP, adenosine 5'-(3-dimethylaminopropyl)triphosphate; GSK3, glycogen synthase kinase 3; RIPK2, receptor-interacting protein kinase 2; PAK4, p21-activated kinase 4; CaMK, calcium/calcmodulin-dependent protein kinase; MARK, microtubule-associated protein-regulating ki-
nase; ERK, extracellular signal-regulated kinase.

Molecular & Cellular Proteomics 6.3 537
Proteomics Analysis of Protein Kinases

To date, the only biochemical techniques permitting the efficient isolation of certain subfractions of the expressed kinase use immobilized low molecular weight kinase inhibitors as selective capture molecules for the affinity enrichment of their cellular target proteins (11–13). Because up to 35 different protein kinases could be identified by the combination of kinase inhibitor affinity chromatography and MS analysis, this type of chemical proteomics strategy might be useful for comprehensive approaches to cellular kinase biochemistry in case the coverage of protein kinases can be substantially increased (14).

Here we report the development of a chromatographic method using four different kinase-selective affinity resins with distinct target specificities. This fractionation procedure efficiently isolates a large variety of protein kinases from total cell extracts and permitted the detection of up to 113 protein kinases per cell line by LC-MS/MS analysis. The established technique is further compatible with phosphoproteomics applications, and more than 200 phosphorylation sites from protein kinases could be assigned in this study.

EXPERIMENTAL PROCEDURES

Cell Culture—Jurkat T lymphoma cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Two days prior to harvest, 20 × 10^6 HEPES-NaOH, pH 7.5, was added to the culture medium. Cells were then grown to a density of about 2 × 10^6 cells/ml, centrifuged by 37 °C, and cells were incubated in the presence of 50 μM calyculin A (Merck) and 0.5 mM sodium pervanadate for 20 min prior to centrifugation and freezing of the cell pellets in liquid nitrogen. Adherent cancer cells were cultivated in Dulbecco’s modified Eagle’s medium (A549 cells) or McCoy’s 5A medium (HCT-116 cells) supplemented with 10% fetal bovine serum and penicillin/streptomycin and grown to confluency on 15-cm dishes prior to cell lysis.

Generation of Kinase Affinity Resins—Bisindolylmaleimide X (Alexis Biochemicals) and AX14596 were dissolved in 50% DMSO, 0.025 M Na₂CO₃, at a concentration of 10 mM. For immobilization, inhibitor solution (1 ml/tube) and 5 n M NaOH (6 μl/tube) were added to washed and drained epoxy-activated Sepharose 6B (0.5 ml/tube), and the coupling reaction was done overnight at 30 °C in the dark under continuous shaking. After two washes with 50% DMSO, 0.025 M Na₂CO₃, remaining reactive groups were blocked with 1 M ethanolamine, pH 11, and the beads were washed as described previously (12, 15). For carbodiimide-mediated coupling, 1 ml of 0.75 mM PP58 (10 mM purvalanol B, from Tocris) dissolved in 50% dimethylformamide (DMF), 50% ETOH was added to 0.5 ml of washed and aspirated ECH-Sepharose 4B (EAH-Sepharose 4B) followed by the dropwise addition of 150 μl of 1 M N-ethyl-N’-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) dissolved in 50% DMF, 50% ETOH. The coupling reaction was done overnight on a rotating wheel at room temperature in the dark. After one wash with 1 ml of 50% DMF, 50% ETOH, a second coupling reaction was done overnight by addition of 33% DMF, 33% ETOH, 34% 1 M ethanolamine (20 mM HAc in 50% DMF, 50% ETOH) and 150 μl of 1 M EDC. Subsequently the beads were washed essentially as described previously (14). All affinity resins were stored in 20% ethanol at 4 °C in the dark.

Cell Lysis and Affinity Chromatography—Frozen pellets of 5 × 10⁹ calyculin/sodium pervanadate-stimulated Jurkat cells were solubilized with 40 ml of high salt lysis buffer containing 50 mM HEPES, pH 7.5, 1 M NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA plus additives (10 mM sodium fluoride, 2.5 mM orthovanadate, 50 ng/ml calyculin, 1% phosphatase inhibitor mixture 1 (Sigma), 1% phosphatase inhibitor mixture 2 (Sigma), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) for 1 h at 4 °C. A549 and HCT-116 cells were lysed with 750 μl of the same buffer per confluent 15-cm dish. Total cell extracts were then sonicated (3 × 5 s) and cleared by centrifugation for 30 min at 20,000 rpm. Supernatants were again sonicated (2 × 5 s), centrifuged for 10 min at 20,000 rpm, and then passed through a 0.45-μm PVDF filter membrane. For affinity chromatography on an AKTA explorer system, four Tricorn 5/20 chromatography columns (GE Healthcare) packed with 0.5 ml of different kinase inhibitor resins were equilibrated to high salt lysis buffer containing 10 mM NaF and 0.1 mM sodium orthovanadate (buffer A). The filtrated cell extract prepared from up to 5 × 10⁹ Jurkat cells or 50 dishes (15-cm diameter) of A549 or HCT-116 cells was passed through four inhibitor columns connected in series, which displayed the immobilized bisindolylmaleimide X (bisX), AX14596, PP58, and purvalanol B (purB) inhibitors as affinity ligands. After loading of the extract, the columns were washed with 17.5 ml of buffer A and then equilibrated to low salt conditions with 12.5 ml of buffer B containing 50 mM HEPES, pH 7.5, 0.15 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, and 0.1 mM sodium orthovanadate. Subsequently the columns were disconnected, and each column was eluted separately with either 1.5 ml (bisX and purB columns) or 2 ml (AX14596 and PP58) of buffer B containing 20 mM MgCl₂, 5 mM AMP-PNP, 5 mM ADP, a 1 mm concentration of the corresponding free inhibitor, and all additives. Fractions of 0.5-ml volume were collected in tubes with 25 μl of prelaid 0.5 mM EDTA solution to complex the magnesium cations present in the elution buffer. With the exception of gefitinib, which was included in the elution buffer for the AX14596 column, the same inhibitors as used for immobilization were added to the specific elution buffers. Subsequently all columns were equilibrated to water and subjected to a second elution step with 2.5 ml 0.5% SDS. Aliquots from the total cell lysate, flow-through, and elution fractions were resolved by SDS-PAGE, and protein detection was done either by silver staining or by immunoblotting with the following antibodies: mouse monoclonal anti-glycogen synthase kinase 3 α/β (GSK3α/β) (Santa Cruz Biotechnology), rabbit polyclonal anti-receptor-interacting protein kinase 2 (RIPIK2) (Affinity BioReagents), and rabbit polyclonal anti-p38 and rabbit polyclonal anti-p21-activated kinase 4 (PAK4) (both from Cell Signaling Technology, Inc.). Protein-containing elution fractions were then concentrated in a vacuum evaporator and then subjected to protein precipitation according to the method of Wessel and Flugge (16).

Protein Digestion and Phosphopeptide Enrichment—Prior to the preparative separation of kinase-enriched protein samples, a 10% aliquot of each elution fraction was resolved by SDS-PAGE. Using 1 μg of BSA as standard protein, the colloidal Coomassie staining technique according to Candiano et al. (17) as protein detection method, and Advanced Image Data Analysis software (FUJI Raytest) for one dimensional quantitation, the composition of each protein fraction was determined. Subsequently the large remainders of the elution fractions containing up to 80 μg of protein were separated on preparative gels. Proteins were resolved on a 1.5-mm-thick SDS gel for no more than 2.5-cm running distance, and gel regions containing no more than 30 visible proteins were excised. Highly intense protein bands were pooled separately, and each lane was divided in two to four pools. Gel pieces from the different pools were processed separately by cutting them into small cubes prior to washing, reduction, alkylation, destaining, and drying as described previously (11). The dried gel pieces were incubated in 50 mM NH₄HCO₃ containing 10% acetonitrile and a protein:trypsin ratio of no more than 50:1 on ice until the gel was completely reswollen. Digestion of protein was performed overnight at 37 °C. Peptides were eluted with 5 × gel
volumes of H$_2$O, then 1% TFA, twice with 40% acetonitrile, 0.1% TFA, and finally overnight and dried in 500-µl portions in a vacuum concentrator.

Before phosphopeptide enrichment using a phosphopeptide enrichment kit (Pierce), all peptides were purified onto RP$_{18}$ material (TopTip 1–10 µl C$_{18}$, SunChrom GmbH or LiChrospher RP18; Merck packed in MobilCol disposable columns; MobilTec). This proved to be absolutely necessary for efficient binding of phosphopeptides onto Ga$^+$$^+$-loaded IMAC beads. Purified peptides were dried into a Speed-Vac and resolubilized for IMAC in 1 µl of binding buffer (methanol: acetonitrile:H$_2$O = 1:1:1 containing 2% acetic acid/µg of peptide, and up to 50 µg were applied to the IMAC beads. The samples were incubated for 10–30 min and resuspended several times during this period. Unbound material was removed by washing the beads up to 10 times using 50 µl of binding buffer. Preconditioning for elution was achieved with 2 × 50 µl of H$_2$O. Elution was done five times with 30 µl of 100 mM NH$_4$HCO$_3$ (pH 9) each. The IMAC elution fractions were combined and dried in a vacuum concentrator, and the eluted peptides were then acidified by drying them down in 50 µl of 0.2% TFA three times followed by a final purification step using ZipTips (Millipore) prior to LC-MS/MS. For subsequent analysis of the phosphopeptide-depleted IMAC flow-through fractions, eluates of two µZipTips (Millipore) were combined per LC-MS/MS experiment.

The separation of the peptide samples was performed using a bionert Ultimate nano-HPLC system (Dionex). 10 µl of each sample (containing up to 1 µg of peptides) were injected, and peptides were purified and concentrated on a C$_{18}$ PepMap precolumn (0.3 mm inner diameter × 5 mm, 100-Å pore size, 3-µm particle size) at a flow rate of 30 µl/min in 0.1% TFA. Subsequently peptides were separated on an analytical 75-µm-inner-diameter × 150-mm C$_{18}$ PepMap column (Dionex, 100-Å pore size, 3-µm particle size) at a flow rate of 200 nl/min. The gradient (solution A: 0.1% formic acid, 5% acetonitrile; solution B: 0.1% formic acid, 80% acetonitrile) started at 5% and ended at 90% B after 30, 60, or 120 min.

LC-MS/MS Analysis and Database Searching—MS and MS/MS data were acquired using a Q-TOFmicro mass spectrometer (Waters, Milford, MA). Doubly and triply charged peptide ions were automatically selected by the MassLynx software and fragmented for a maximum of 18 s per peptide. MS data were automatically processed, and peak lists for subsequent protein identification by database searches were generated using MassLynx version 4.1b. Database searches were carried out with Mascot 2.1.3 and Mascot Daemon 2.1.3 in the UniProtKB/Swiss-Prot database (release 49.4 of April 4, 2006 with 215,741 entries; taxonomy: Homo sapiens with 13,745 entries). Proteins were only accepted as identified when at least one unique peptide showed an individual score above 30, which indicated identity or extensive homology (p < 0.05) using the given settings (enzyme, trypsin; maximum missed cleavages, 2; fixed modification, carbamidomethyl (Cys); variable modifications, phosphorylation (Ser, Thr, Tyr); peptide tolerance, 50 ppm; MS/MS tolerance, 0.15 Da).

**RESULTS**

**Development of a Protein Kinase-selective Prefractionation Strategy**—Because previously described kinase prefractionation methods have used immobilized, ATP-competitive inhibitors as selective ligands for affinity chromatography with some success, we reasoned that further method optimization and the combined use of inhibitor matrices with distinct selectivity profiles might permit the enrichment of a substantial fraction of cellular protein kinases (12, 14, 15, 18, 19). Initially we evaluated the potential of different kinase inhibitor resins for this type of application, and test runs using total cell extracts loaded onto single inhibitor columns revealed that the amount of specifically retained protein decreased in the order of purB $>$ PP58 $>$ AX14596 $>$ bisX as immobilized affinity ligands (data not shown). Based on these results, we arranged the four different inhibitor columns in ascending order with respect to their protein binding capacities (Fig. 1). Thus, for example, purB-interacting protein kinases with affinity to any of the other immobilized ligands would already be retained prior to reaching the purB column, thereby resulting in a reduced complexity of the purB-bound protein mixture and a more even kinase fractionation to the four different resin eluates. To test this prefractration strategy, total cell extract prepared from A549 lung adenocarcinoma cells was loaded on the four different inhibitor columns connected in series (Fig. 1). The lysis buffer contained 1 M NaCl to facilitate the efficient solubilization of protein kinases localizing to the nucleus or to insoluble cytoskeletal components. Moreover various inhibitors of serine/threonine and tyrosine protein phosphatases were included to preserve cellular protein phosphorylation after cell lysis. After sample loading and subsequent washing to remove unbound material, the four columns were disconnected from each other, and specifically retained material was released by separate elution of each column with buffer containing the corresponding free inhibitor plus ADP and the non-hydrolyzable ATP analogue AMP-PNP (Fig. 1). Thus, in contrast to earlier procedures (12, 14), ATP was omitted from the elution buffer to prevent any potential *in vitro* phosphorylation events during the subsequent processing of the enriched protein kinase fractions. Analysis of the total cell lysate, flow-through, and elution fractions by SDS-PAGE and silver staining revealed distinct patterns of eluted proteins for each of the four columns (Fig. 2). Importantly because relative to the initial cell extract and the flow-through, 5000-fold larger portions of the elution fractions were analyzed by silver staining, the fractionated proteins represented highly enriched subfractions of the cellular proteome (Fig. 2, *upper panels*). To analyze the fractionation of known protein kinase targets of the immobilized inhibitors, we further subjected the lysate, flow-through, and elution fractions to immunoblotting with selective antibodies. As shown in Fig. 2, most of GSK3α/β was retained by the bisX column and therefore did not appear in the eluate from the purB resin, which is also known to bind these two quite abundant protein kinases (19). Similar observations were made for RIPK2, which was depleted from the extract by immobilized AX14596 and was not detected in the eluate from the succeeding PP58 column with comparable RIPK2 binding properties (12, 14). As further seen in Fig. 2, the protein kinases p38 and PAK4, which are known protein kinase targets of PP58 and purB, were retained by the respective inhibitor columns (14, 19). Taken together, these results established a multicolumn separation strategy permitting the fractionation of kinase inhibitor-interacting proteins into four different, highly enriched subfractions derived from the initial total cell lysate.

**Protein Kinase Identification from Different Cell Lines**—Next we used this enrichment technique to fractionate total cell
lysates from different cancer cell lines prior to analysis by tandem mass spectrometry. As starting materials, we used total extracts prepared from A549 lung adenocarcinoma, HCT-116 colon carcinoma, and phosphatase inhibitor-treated Jurkat T lymphoma cells. After chromatographic separation, the fractions containing the majority of purified protein were pooled for each of the four inhibitor columns, and the eluates from the bisX, AX14596, PP58, and purB resins were then separately resolved by SDS-PAGE. Gel slices covering different molecular weight regions were excised and subjected to in-gel tryptic digestion, and the eluted peptides were then further fractionated with IMAC beads to prepare phosphopeptide-enriched and -depleted fractions (Fig. 1). Subsequent LC-MS/MS analysis of all peptide fractions and assignment of the acquired spectra by the Mascot search algorithm led to the identification of more than 140 different members of the protein kinase superfamily in the three investigated cell lines (Supplemental Table 1). Many protein kinases were retained by more than one inhibitor column, albeit a significantly higher Mascot score often indicated the preferential fractionation to one of the column eluates in most cases (Supplemental Table 2). In addition, about 200 other proteins including different nucleotide-binding enzymes and some regulatory subunits of protein kinases were also detected in the fractions enriched with the four inhibitor resins (Supplemental Table 2). As shown in Fig. 3A, the majority of confidently assigned proteins from the PP58 and purB column eluates represented protein kinases, whereas the bisX and particularly the AX14596 resins were found to retain higher numbers of other proteins. However, because in contrast to the protein identifications about 80% of the confidently assigned peptides matched to protein kinases, the overall fractionation procedure appeared to be highly kinase-selective in terms of protein amount. As illustrated for the protein kinases enriched and detected in this study, the more than 140 identified members of the human protein kinase superfamily were quite evenly distributed in the phylogenetic tree generated by Manning et al. (1), indicating no major sequence-related bias of our multicolumn prefractionation procedure for a specific group of protein kinases (Fig. 3B). Moreover as depicted for the tyrosine kinase group in Fig. 3C, LC-MS/MS analysis of the affinity column eluates led to the identification of different sets of protein kinases from the Jurkat, HCT-116, and A549 cells. Although the experimental procedures used in this study do not allow the quantitative comparison of kinase expression profiles, a variety of cytoplasmic tyrosine kinases specific for hematopoietic

Fig. 1. Overview of the experimental strategy. For the enrichment of protein kinases by affinity chromatography, total cell lysate was first passed through four consecutive columns, which contained affinity matrices with structurally different protein kinase inhibitors as immobilized capture ligands. The small molecule inhibitors bisindolylmaleimide X, AX14596, and PP58 were covalently coupled via the circled primary amino groups to Sepharose resin, whereas purvalanol B was immobilized through the indicated carboxyl moiety. Upon sample loading and the subsequent washing steps, the four affinity columns were disconnected from each other, and retained proteins were released by separate, column-specific elution procedures. After gel electrophoresis and tryptic digestion of the protein present in the different eluates, phosphopeptides were separated by IMAC prior to the separate LC-MS/MS analysis of phosphopeptide-enriched and -depleted fractions. The acquired MS/MS spectra were then interpreted with the Mascot database search algorithm.
cells (LCK, BLK, and ZAP70) were only found in Jurkat cell lysate, whereas certain receptor tyrosine kinases expressed in adherent cells (epidermal growth factor receptor, Met, and Ron) were only detected in the column eluates prepared from the cancer cell lines A549 and HCT-116. Thus, our data demonstrate that the multicolumn procedure permitted the detection of several times more members of the human kinase than in any previous proteomics study using immobilized kinase inhibitors (14). In addition, the results obtained also provide qualitative insights into major differences of kinase expression between different cell types.

Phosphoproteomics Analysis of Protein Kinases—The efficient enrichment of 140 protein kinases from the vast majority of other cellular proteins provided an ideal starting point for the characterization of their phosphorylation sites. For further phosphopeptide analyses, we performed IMAC separation of the tryptic digests prepared from eluates of the different kinase affinity columns (Fig. 1). LC-MS/MS analyses and subsequent Mascot searches against UniProtKB of the phosphopeptide-containing IMAC eluates identified in total 966 protein kinase-derived phosphopeptides with identification scores higher than 20. The phosphopeptide enrichment efficiency was always better than 90%, and thus any further chemical treatment like methylation of acidic peptides could be omitted. About 210 different non-redundant phosphopeptides derived from the highest scoring spectra of each individual peptide were selected for systematic manual validation to confirm their identities and to assign the exact phosphorylation site(s). 14 of these spectra had to be rejected as a potential phosphopeptide, and the site of modification could not be determined in a further 11 cases.

The analysis of Jurkat cells, which were treated with phosphatase inhibitors to enhance cellular phosphorylation levels, led to the identification of 128 different high confident phosphopeptides from protein kinases. In adherent A549 and HCT-116 cancer cells lines, which were cultivated in normal growth media without protein phosphatase inhibitors, 67 and 86 different protein kinase-derived phosphopeptides could be assigned, respectively. In total, we could identify 209 different phosphorylation sites that could be assigned unambiguously at 185 different high confident phosphopeptides from protein kinases; about half of those were found in more than one cell line (Supplemental Table 3). It is noteworthy that with 94 of 209 phosphorylation sites more than 45% have not been described previously. The peptides from kinases harboring new phosphorylation sites are shown in Table I. New phosphosites were checked thoroughly using the databases Swiss-Prot, Phospho.ELM, PhosphoSite, and the recently published Phosida database (20). A full list of all protein kinase phosphopeptides, including all their peptide views from the Mascot results sheet, found in this study is provided in Supplemental Table 4. Importantly this dataset includes the in-

Fig. 2. Protein kinase fractionation and enrichment by multicolumn affinity chromatography of lysate from A549 cells. To visualize the proteins specifically enriched by the different inhibitor affinity, 2% of the elution fractions from the bisX, AX14596, PP58, and purB columns were compared with 0.0004% aliquots of the total lysate and flow-through by SDS gel electrophoresis and silver staining (upper panels). In parallel, 0.3% of the elution fractions from each specific column elution were separated together with 0.02% aliquots of the lysate and flow-through. After transfer to nitrocellulose membrane, immunoblotting was performed with specific antibodies recognizing GSK3, RIPK2, p38, and PAK4 as indicated. CSFR, colony-stimulating factor receptor; EGFR, epidermal growth factor receptor; Eph, ephrin receptor; FAK, focal adhesion kinase; FGFR, fibroblast growth factor receptor; FRK, Fos-regulatory kinase; InsR, insulin receptor; IRR, insulin receptor-related; JAK, janus kinase; PDGFR, platelet-derivied growth factor receptor; ROR, regeneron orphan receptor; SYK, spleen tyrosine kinase; TIE, tyrosine kinase with immunoglobulin and EGF, repeats.
depth analysis of a variety of poorly characterized members of the protein kinase superfamily such as for example calcium/calmodulin-dependent protein kinase 2δ (CaMK2δ), NEK9, microtubule-associated protein-regulating kinase 2 (MARK2), and PCTAIRE2, and to our knowledge represents one of the largest collection of phosphorylation sites on protein kinases reported to date. Furthermore we used synthetic phosphopeptides to validate the assignment of phosphorylation sites of particular interest. As shown in Fig. 4, masses and relative intensities of fragment ions found in the MS/MS spectra for two synthetic phosphopeptides matched the ones acquired during the course of our LC-MS/MS experiments of kinase-enriched samples. Interestingly according to predictions by the computer algorithm SCANSITE, one of the phosphopeptides harbors a putative substrate site for CDK5-mediated phosphorylation in BRAF, which lies in close proximity to the Ras binding domain of this kinase and was identified in all three cell lines (Fig. 4). The other site fulfills the sequence criteria for extracellular signal-regulated kinase 1 (ERK1)-mediated phosphorylation and is present on a GCN2 phosphopeptide identified from all three cell lines (Fig. 4). Thus, the identification of new phosphosites on protein kinases can provide hints for their functional regulation by other members of this enzyme superfamily and thereby warrant further experimental verification of these potential relationships.

**DISCUSSION**

Due to their key regulatory functions in cell signaling, the members of the protein kinase superfamily represent one of the most information-rich parts of a cellular proteome. The biochemical characterization of protein kinases usually requires an efficient enrichment step prior to their analysis by MS methods. Typically antibody-mediated affinity purification is used to study protein kinases either on a one-by-one basis or in the context of tyrosine phosphorylated cellular proteins (21–25). However, these methods do not permit the analysis of large numbers of
| Kinase | Protein name | Sequence | Site(s) $^d$ | A549 | HCT-116 | Jurkat |
|--------|--------------|----------|-------------|------|---------|--------|
| Arg    | ABL2_HUMAN   | STSLSGGLSGLPEQDCR | S783 |      |         | X      |
| Arg    | ABL2_HUMAN   | VSSTSSQPEENVDR | S817 |      |         | X      |
| Arg    | ABL2_HUMAN   | VCPLSPTLTK | S936 |      |         | X      |
| BRAF   | BRF1_HUMAN   | ALGKSLGPOQR | S419 | X    | X       | X      |
| BRAF   | BRF1_HUMAN   | SNKSPPOKPIVR | S151 | X    | X       | X      |
| CaMK2δ | KCC2D_HUMAN  | NFSAAKSLKLK | S319, S323 |                |         | X      |
| CaMK2γ | KCC2G_HUMAN  | NFSAASKLNLKK | S315, S319 |                |         | X      |
| CaMK4  | KCC4_HUMAN   | DPIQDQGNDMK | S360 |      |         | X      |
| CaMK4  | KCC4_HUMAN   | LGSASSSSHSQCESHK | S341 |      |         | X      |
| CDK10  | CDK10_HUMAN  | AGYVGVKTPMTPK | T196 | X    | X       | X      |
| CK1δ   | KC1D_HUMAN   | GAPVNISSSDLTGR | S382 | X    |         | X      |
| CK1δ   | KC1D_HUMAN   | GLPSAGSGRLR | S331 | X    | X       | X      |
| CK1ε   | KC1E_HUMAN   | GAPANVSSDLTGR | S389 |      |         | X      |
| CLK3   | CLK3_HUMAN   | YRVEPEPDPYLSRY | S9 | X    | X       | X      |
| EphA2  | EPHA2_HUMAN  | ARQSPEDVYFSK | S570 |      |         | X      |
| EphA2  | EPHA2_HUMAN  | LPSTSGSEGVPFR | S897, T898, S901 |                |         | X      |
| EphB2  | EPHB2_HUMAN  | FLEDDTDSPTYTSALGK | T775 |      |         | X      |
| Fer    | FER_HUMAN    | YIQENDEKEPPVVNEEDAR | Y402 | X    | X       | X      |
| Fyn    | FYN_HUMAN    | DGSNGSSGYR | S20 |      |         | X      |
| GCK    | M4K2_HUMAN   | TQPSEIGFPQVK | S327 |      |         | X      |
| HKKZC1 | M4K4_HUMAN   | KGSSVNQNVPTNTFSQDSR | S900 |      |         | X      |
| HKKZC1 | M4K4_HUMAN   | LVDGFVSSQDLRTVR $^d$ | T181 | X    |         | X      |
| HKKZC1 | M4K4_HUMAN   | RDSPLQGSQQSQSQAQR | S639 |      |         | X      |
| HKKZC1 | M4K4_HUMAN   | SEIFSNPNSVSHPLQRAPAEPQVPVR | S602 |      |         | X      |
| HPK1   | M4K1_HUMAN   | AGNPIAHSPHR | S566 |      |         | X      |
| HPK1   | M4K1_HUMAN   | FRSPIDPGGMSGGDLQGPLVR | S407 |      |         | X      |
| HPK1   | M4K1_HUMAN   | FRSPIDEPGSMSGGDLQGPLVL | S413, S421 |      |         | X      |
| HPK1   | M4K1_HUMAN   | KQLSSEQDYYDDVDIIPTIPADTDPPPLP| S374, S376, Y381 |      |         | X      |
| ITK    | ITK_HUMAN    | SNEVEMEDISTGFR | S565 |      |         | X      |
| KHS1   | M4K5_HUMAN   | TASENF2NQLQFEPLRK | S335 | X    |         | X      |
| Lok    | LOCK_HUMAN   | ESESTAGFSDSLVR | S161 |      |         | X      |
| Lok    | LOCK_HUMAN   | HTYNSQDLCTR | S212 |      |         | X      |
| LIMK1  | LIMK1_HUMAN  | SCDRSRPGAGLGPASOR | S208, S310 |                |         | X      |
| LIMK2  | LIMK2_HUMAN  | SNLRKSPGSPPSPEPLLFR | S287, S291, S298 |                |         | X      |
| LIMK2  | LIMK2_HUMAN  | SGPSPPSPEPLLFSR | S203 |      |         | X      |
| MARK1  | MARK1_HUMAN  | RGTGISGSK | S649 | X    |         | X      |
| MARK2  | MARK2_HUMAN  | DQQLPVPVTAPSFIVSQQR | S619 | X    | X       | X      |
| MARK2  | MARK2_HUMAN  | GRKSTASADEPQHGRYR | S40 |      |         | X      |
| MARK2  | MARK2_HUMAN  | KTPSTPNTSISLSTSNR | T469 | X    |         | X      |
| MARK2  | MARK2_HUMAN  | RAGGAEFISK | S631 |      |         | X      |
| MARK2  | MARK2_HUMAN  | TTPSPTNHLSTSNTR | S479 |      |         | X      |
| MARK2  | MARK2_HUMAN  | VPPSAPSAHNNSSSGGAPDR | S571 |      |         | X      |
| MARK3  | MARK3_HUMAN  | GIAFASPLMLGNASPNK | S492 | X    |         | X      |
| MARK3  | MARK3_HUMAN  | RYSIMHAGPAIPSVYYPPK | S442 | X    |         | X      |
| MARK3  | MARK3_HUMAN  | SROSTNLSK | S624 |      |         | X      |

New phosphorylation sites identified on protein kinases

CK1, casein kinase 1; CLK3, Cdc2-like kinase 3; EphA2, ephrin receptor A2; LIMK, LIM domain-containing kinase; PKC, protein kinase C; PKD2, protein kinase D2; RSK, ribosomal protein S6 kinase; TNK2, Traf2 and NCK interacting kinase; ULK1, Unc-51-like kinase 1.
protein kinases from different groups and families of the human kinome. To address this issue, we have developed a prefractionation method that uses different immobilized kinase inhibitors as ligands for kinase affinity chromatography, and up to 113 protein kinases per cell line could be identified by LC-MS/MS after their separation from the overwhelming majority of other cellular proteins. The multicolumn prefractionation procedure resulted in a substantially increased coverage of the expressed kinome compared with previous chemical proteomics studies with individual affinity resins (12, 14, 15). Moreover the immo-

| Table I—continued |
|-------------------|
| MARK4      | MARK4_HUMAN | §SDKGPSWSSR | S26 | X |
| Met        | MET_HUMAN   | VHTPLHDR    | T977 | x | x |
| MST1       | STK4_HUMAN  | §PQDDYFLK   | Y533 | x |
| Myt1       | PMYT1_HUMAN | §SFPFGPK     | S143 | x |
| Nek9       | NEK9_HUMAN  | §STVTEPIAVTSR | S332 | x |
| Nek9       | NEK9_HUMAN  | §STVTEPIAVTSR | T333 | x |
| Nek9       | NEK9_HUMAN  | §STPQLDVIK   | T358 | x |
| PAK4       | PAK4_HUMAN  | §RKPLSGDVTGTPQAPLAGSAK | S161 | x | x | x |
| PAK4       | PAK4_HUMAN  | §EGGGPQESSRDKPLSGDVTGTPQAPLAGSAK | S167 | x |
| PAK4       | PAK4_HUMAN  | §FAHSEAGGGGSRD | S148 | x |
| PAK4       | PAK4_HUMAN  | §LAGRPFNTTPR | T207 | x |
| PAK4       | PAK4_HUMAN  | §RDSPPPPPAR  | S104 | x | x | x |
| PAK4       | PAK4_HUMAN  | §RPLSPGDVTGTPQAPLAGSAK | S183 | x |
| PAK4       | PAK4_HUMAN  | §NSLRRDSPPPAR | S104 | x | x | x |
| PAK4       | PAK4_HUMAN  | §NSLRRDSPPPAR | S99, S104 | x | x |
| PAK4       | PAK4_HUMAN  | §SPVPRQ      | S291 | x |
| PCTAIRE1   | PCTK1_HUMAN | §LTNQSPFKPLSR | S138 | x | x |
| PCTAIRE1   | PCTK1_HUMAN | §RLSLPADRLPGLEK | S119 | x | x |
| PCTAIRE2   | PCTK2_HUMAN | §RAILSESIFGK | S180 | x |
| PCTAIRE2   | PCTK2_HUMAN | §RISMEDLNK   | S137 | x |
| PCTAIRE2   | PCTK2_HUMAN | §RLTLRL      | S9    | x | x |
| PCTAIRE3   | PCTK3_HUMAN | §RAILSDLQGFK | S130 | x |
| PCTAIRE3   | PCTK3_HUMAN | §RFSLSVPR    | S12   | x |
| PCTAIRE3   | PCTK3_HUMAN | §RFSMEDVSK   | S87   | x |
| PKGα       | KPCA_HUMAN  | §TFCGTSDYPAEIAYQYPYK | T496, T500 | x |
| PKCθ       | KPCT_HUMAN  | §ALINSMDIINMR | S685 | x | x |
| PKD2       | KPCD2_HUMAN | §LGTSGLPCEDAEASLR | S214 | x |
| PKN3       | PKN3_HUMAN  | §RGPSPSAPSTR | S544, S549 | x |
| RIPK2      | RIPK2_HUMAN | §SPSGLLNQNK  | S531 | x |
| RSK1/p90RSK| KS6A1_HUMAN | §TPKDSGTPPSAGAHQFLR | T359, S363, S369 | x |
| RSK2       | KS6A3_HUMAN | §KAYSFGCTVEYAMEVPR | S227, T231 | x | x |
| RSK2       | KS6A3_HUMAN | §NQSDLEVLYEPVR | S715 | x | x | x |
| Src        | SRC_HUMAN   | §LFGFNSDVTSPQR | S68 | x |
| TNIK/ZC2   | TNIK_HUMAN  | §GNSDPTSEPPLPSTR | S640 | x |
| TNIK/ZC2   | TNIK_HUMAN  | §TSSISAPLAR   | S680 | x |
| ULK1       | ULK1_HUMAN  | §TPSGLNLALLAR | S638 | x |
| Wee1       | WEE1_HUMAN  | §SPRPDPGTTPPHK | T173 | x |
| ZAK        | MLTK_HUMAN  | §SSPPTQVGLTK  | S637 | x | x | x |

*a* Protein kinase name according to the nomenclature by Manning et al. (1).

*b* Name of the protein entry in the UniprotKB/Swiss-Prot database.

*c* All phosphorylated residues identified in the phosphopeptides are highlighted.

*d* Positions of the identified phosphorylated residues; new phosphorylation sites are in bold letters.

*e* Identical peptide sequence also present in MINK/ZC3 and TNIK/ZC2.
Bilized kinase inhibitor purB revealed surprising potential as an efficient prefractionation tool. Although it was used as the last capture reagent for kinases not retained by any of the three other inhibitor resins, elution fractions from the purB resin were found to contain significantly more protein kinases than identified in previous studies (18, 19).

Fig. 4. MS/MS spectra of selected phosphopeptides. Shown is a comparison of MS/MS spectra acquired for cellular GCN2 (HERPAG-PGpTPPPDSPGPLAK where pT is phosphothreonine) and BRAF (SNPKpSPQPIVR where pS is phosphoserine) phosphopeptides and their synthetic counterparts. Fragment ions resulting from the loss of phosphoric acid (~98 Da) are marked by asterisks.
Although the methodology described in this study represents a substantial advancement over previously published procedures, further method optimization by including additional or even more unselective kinase inhibitors as affinity ligands can be envisioned and would further increase protein kinase coverage. Alternatively for specific application relating to the analysis of specific kinase subsets retained by a single resin, the experimental effort can be reduced by performing affinity chromatography on a single column. For example, purB chromatography would be suitable for the simultaneous phosphoproteomics analysis of the CDK family members expressed in a cell line or tissue, whereas immobilized PP58 might be particularly useful for the parallel analysis of the ERK, JNK (c-Jun NH₂-terminal kinase), and p38 mitogen-activated protein kinases as well as several of their upstream activators (such as the kinases MEK1, MEK2, MEKK1, or BRAF). Thus, the current dataset provides a framework for the adjustment and modification of kinase-selective prefracination strategies according to the specific experimental situation.

As shown in this study, protein kinase prefracination combined with selective phosphopeptide enrichment permitted the identification of both known and novel phosphorylation sites on the isolated protein kinases by LC-MS/MS analysis. The information about previously unknown phosphorylation sites on protein kinases provides specific rationales for further mutational studies to evaluate potential regulatory functions with respect to cellular kinase activity control. In addition, we believe that the phosphoproteomics analysis of highly enriched protein kinases reported in our present study is likely to have a considerable potential when combined with quantitative MS methods based on, for example, metabolic labeling with distinct isotope variants of arginine and lysine or peptide modification with stable isotopes. The results of our study strongly support the hypothesis that phosphoproteomics analysis of the developing mouse brain will significantly facilitate monitoring of relevant phosphorylation events directly on the central regulators of cellular signal transduction networks and thereby provide highly informative insights into the signaling state of biological systems.

Acknowledgments—We are very grateful for the generous support provided by Prof. Axel Ullrich that was essential to conduct this study. PPS8 was kindly provided by GPC Biotech AG. The kinome dendrogram displayed in Fig. 3 was reprinted with permission of Cell Signaling Technology, Inc.

* This work was supported in part by the Max Planck Institute of Biochemistry, Department of Molecular Biology (Director: Prof. Axel Ullrich), and by the German Bundesministerium für Bildung und Forschung (BMBF) “Verbundvorhaben: Intergenomics-Bioinformatische Modellierung der Wechselwirkung von Genomen” (Grant 031U110A/031U210A). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[§] The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.

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