Approaches to Define Antigen Receptor-induced Serine Kinase Signal Transduction Pathways*

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In the present report we describe the properties of a novel phospho-specific antiserum that has opened a route to the characterization of antigen receptor-activated serine kinase pathways in lymphocytes. The basis for the present work was that Ser-21 in glycogen synthase kinase 3α is robustly phosphorylated following antigen receptor triggering. We predicted accordingly that antigen receptors would also stimulate phosphorylation of other proteins with a similar sequence. To test this idea we raised an antibody against the phosphopeptide RARTSpSFAP, where pS is a phospho-serine corresponding to the glycogen synthase kinase 3α Ser-21 sequence. The resulting antiserum was called phospho antibody for proteomics-1 (PAP-1). The present study describes the properties of PAP-1 and shows that it can reveal quite striking differences in the phospho-proteome of different cell types and is able to pinpoint new targets in important signal transduction pathways. PAP-1 was used to map protein phosphorylations regulated by the antigen receptor in T cells. One of these PAP-1-reactive proteins was purified and revealed to be a previously unrecognized target for antigen receptor signal transduction, namely an “orphan” adapter SLY (Src homology 3 (SH3) domain-containing protein expressed in lymphocytes). The use of sera detecting specific phosphorylation sites is thus proved as a powerful method for the discovery of novel downstream components of antigen receptor signals in T cells.

The T cell antigen receptor (TCR)† is the key to T cell activation and controls the specific immune response. An immediate consequence of triggering the TCR is activation of cytosolic protein-tyrosine kinases of the Src, Syk, and Tec families (1). These tyrosine kinases phosphorylate an array of adapter molecules that initiate a cascade of signaling pathways (2,3). The key events are the activation of Ras and Rho family GTPase signaling networks and the metabolism of inositol phospholipids, which regulate both intracellular calcium and the activity of diverse serine/threonine kinases. The serine kinases triggered by antigen receptors include members of the protein kinase C (PKC) and D (PKD) kinase family, phosphatidylinositol-3-kinase (PI3K)-controlled serine kinases such as protein kinase B (PKB) and the Raf-1/Mek/Erk1,2/Rsk kinase cascades (4–7).

T cell activation requires a period of sustained signaling, and it is during this sustained response to antigen receptor engagement that serine kinase pathways are active (8–10). The action of antigen receptor-regulated serine kinases is essential for T cell activation, but there is little information about their substrates in T cells that can explain their actions. Some progress in understanding this action may be gained by looking for the existence of evolutionarily conserved serine kinase pathways in T cells. For example, Erk1/2 phosphorylation of the ternary complex transcription factors Elk1 (11), PI3K/PKB- or PKC-mediated phosphorylation and inactivation of glycogen synthase kinase 3α and 3β (GSK3α/β) (9, 12), and PI3K/PKB-regulated phosphorylation of Forkhead family transcription factors (13–15) are all conserved responses that have been described in T cells. However, such an approach ignores the possibility that ubiquitous kinases might have unique T cell-restricted substrates. Lack of knowledge about serine kinase substrates is a bioinformatic problem common in many cell systems. Phospho-amino acid analysis of the proteome shows that serine phosphorylation is one of the most common post-translational events. However, reliable identification of serine kinase targets in intact cells has proved problematic and is a major rate-limiting step in furthering our knowledge and understanding of signal transduction pathways in eukaryotic cells.

In the last 10 years, some useful biochemical and genetic techniques have been developed to search for the downstream targets of serine kinases. These are largely based on the use of purified serine kinases to find substrates in cell lysates (16) or in cDNA expression library screening (17). Biochemical strategies require relatively pure serine kinase preparations of high specific activity and have the limitation that they examine the substrate specificity of a kinase free of any spatial restraints that might limit substrate availability in an intact cell. A recent breakthrough in the analysis of serine kinase networks came with the production of specific antisera against defined serine or threonine phosphorylated peptides that could then be
used to map phosphorylation of known proteins as cells respond to external stimuli.

A particular protein kinase will have a theoretical optimal substrate that is determined by its shape in relation to the reaction pocket of the kinase. Because this is partly determined by the local residues around the target serine, it has been possible to work out optimal amino acid sequences for protein kinases using in vitro assays of a degenerate peptide library. From this candidate, substrate sites of serine/threonine kinases have been identified and used to establish a protein data base motif-scanning program termed Scansite (18–20). There are thousands of potential serine/threonine kinase sites within the proteome, and it is still very difficult to predict and map pathways of serine phosphorylation by data base analysis alone. In vitro experimental data is still needed to narrow down potential targets to a reasonable level. In this context, one useful way to obtain biochemical data to facilitate computer-based searches for serine kinase targets is to use antibodies raised against phosphorylated peptide candidate sites (21).

The immunoreactivity of phospho-specific antisera is determined not only by the presence of a phosphorylated amino acid but also by the charges and hydrophilic or hydrophobic properties of surrounding amino acids. Phospho-specific antisera thus have the potential to cross-react with proteins that contain a phosphorylated peptide that is not necessarily identical in terms of linear sequence but that is structurally similar. The potential cross-reactivity associated with the use of phospho-specific antisera is a feature that can be exploited to delineate serine kinase networks in cells. In this context, antibodies reactive with phosphorylated PKB and PKC substrate sequences have been used recently to define new sites of serine phosphorylation in intact cells (21–23).

The present study describes the properties of an antisera termed PAP-1 (phospho antibody for proteomics-1) that recognizes a tier of proteins regulated by serine phosphorylation in activated cells. PAP-1 was raised against the phospho-peptide RARTSpSFAEP corresponding to Ser-21 in glycogen synthase kinase 3α, a known substrate for Akt/PKB. PAP-1 recognizes a subset of PKB substrates and can be used to identify novel components of PKB signaling. However, the immunoreactivity of PAP-1 is quite distinct to that of antisera raised against the PKB substrate sequence RXRXXpSer. In T lymphocytes, the T cell antigen receptor is known to regulate the activity of diverse serine kinases, but analysis of the complex patterns of serine phosphorylation in activated T cells has been a laborious and slow process. In the present work, PAP-1 was used to map protein phosphorylations regulated by the antigen receptor in T cells. One of these PAP-1-reactive proteins was purified and revealed to be a previously unrecognized target for antigen receptor signal transduction, namely an “orphan” adapter SLY (Src homology 3 (SH3) domain-containing protein expressed in lymphocytes). PAP-1 is thus able to identify novel downstream components of antigen receptor signal transduction in T cells.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents—**Phospho-peptides synthesized by Dr. Nicola S. Reiley and colleagues (Peptides Laboratory, Cancer Research UK) were coupled to keyhole limpet hemocyanin (Calbiochem, Nottingham, UK) and injected into rabbits at Murex (Darford, UK). PAP-1 antiserum was raised against RARTSpSFAEP, where pS denotes a phospho-serine. PAP-1 was diluted in an equal volume of glycerol and stored at −20 °C. Antibodies that recognized phospho-Ser-473 PKB, pan PKB, phospho-Ser21/9 GSK3 α/β, phospho-Ser-256 FKHR, and the phospho-substrate antibodies (PKB phospho-substrate, phospho-14-3-3 binding motif, and phospho-Ser-Thr-Phe) were purchased from New England BioLabs (Beverly, MA). The pan GSK3 and phospho-Thr24 FKHRL1 antibodies were purchased from Upstate Biotech Inc. (Lake Placid, NY). The IL-16 antibody was from R&D Systems (Abingdon, UK). The phospho-ERK1/2 antibody was from Promega (Southampton, UK). The Hybridisation development unit of Cancer Research UK purified the anti-CaMKII UCHT1 and 9E10 (anti-Myc) antibodies. Horseradish peroxidase-coupled donkey anti-rabbit and sheep anti-mouse antibodies were obtained from Amersham Biosciences (Little Chalfont, UK). Silver staining was performed using the Owl kit (Cambridge Biosciences). Materials for protein purification, including all columns and electrofocusing reagents, were obtained from Amersham Biosciences.

**DNA Constructs—**The cDNA encoding myc-tagged SLY in the pSecTag vector was kindly provided by Sandra Beer (Institute of Medical Microbiology, Munich, Germany). The phosphorylation sites SLY (Ser-27) was mutated to alanine, following the manufacturer's recommendation using the QuickChange kit from Stratagene. The point mutations were confirmed by sequencing.

**Cell Culture and Cell Stimulation—**T lymphocytes were prepared from peripheral blood mononuclear cells isolated from healthy donors. Human T lymphoblasts were generated as described previously (24) and maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, penicillin, streptomycin, and IL-2 (20 ng/ml) (Chiron, Harefield, UK) at 37 °C and 5% CO2. After 2 weeks of growth, lymphoblasts were restimulated by washing in medium and allowing to rest in RPMI 1640 with 10% FCS in the absence of IL-2 for 48 h. The mouse B lymphoma cell line A20 were cultured in RPMI 1640 supplemented with 5% FCS, 2 × 10−5 M β-mercaptoethanol, penicillin, and streptomycin.

The PKB-ER retinal pigment epithelial (RPE) cells (Clontech, Cowley, Oxford, UK) were kindly provided by Dr. Julian Downward and Dr. Subham Basu (Cancer Research UK London Research Institute). The cells were cultured in Dulbecco’s modified Eagle’s medium-Ham’s F-12 supplement with 2 mM L-glutamine, 0.348% sodium bicarbonate, and 10% FCS.

Rested T-lymphoblasts were stimulated at 2 × 106 cells per milliliter in RPMI for 10 min, unless otherwise mentioned, with either 50 ng/ml PDBu (Calbiochem, Nottingham, UK) or 10 μg/ml of the anti-CD3 antibody UCHT1. Similarly, A20 cells were stimulated with either 10 μg/ml (Fab′)2 rabbit anti-mouse antibody (Zymed Laboratories Inc., Cambridge, UK) or a peroxidase-conjugated antibody of 1 μg/ml of 1 μM α-Neu5Ac, 1 mM peroxide. Cells were washed in cold PBS pelleted and frozen at −80 °C before being lysed. When required, cells were preincubated. For Western blot analysis, the following inhibitors for 30 min, either PD98059 (New England BioLabs) or LY294002 (BIOMOL Research Laboratories, Plymouth Meeting, PA). RPE cells were grown in 100-mm tissue culture dishes and treated with 100 ng/ml tamoxifen for 4 h or 50 ng/ml PDBu for 5 min, washed in cold PBS, and lysed in 1 ml of lysis buffer per dish.

**Transient Transfection—**A20 cells were electroporated as previously described (25). Briefly, in 4-mm electroporation cuvettes 107 cells and the indicated quantity of plasmid DNA were resuspended in 500 μl of RPMI medium and submitted to one pulse of 310 mV at 960 microfarads in a Bio-Rad electroporator yielding a time constant of 19–21 ms. A20 cells were then resuspended in complete medium at 107 per ml and left to recover for 12 h.

**Cells Extracts and Immunoprecipitation—**T-cells (107), A20 cells (106), or RPE cells (2 × 106) were lysed in 0.5 ml of lysis buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM HEPES, pH 7.4, 10 mM NaF, 10 mM iodacetamide, 2 mM β-glycerophosphate, 1 mM EDTA, 100 μM sodium orthovanadate, 1 μM phenylmethylsulfonyl fluoride (PMSF), and the small peptide inhibitors apropin, leupeptin, pepstatin A, and chymostatin, all at 1 μg/ml. Whole cell lysates were cleared at 14,000 rpm for 10 min, and acetone was precipitated before being resuspended in reducing sample buffer as previously described (26). For immunoprecipitation, the cleared lysates were transferred to a fresh tube tube containing 20 μl of protein G-agarose beads (Amersham Biosciences) and precleared at 4 °C for 15 min before being centrifuged at 5000 rpm for 5 min. The supernatant was then transferred to a fresh tube containing 10 μl of anti-IL-16 antibody at 0.5 μg/ml in phosphate-buffered saline. After 1 h of gentle agitation at 4 °C, 20 μl of protein G-agarose beads was added to each sample and incubated for a further 2 h. Beads were washed three times as before with PBS, and the acetone was precipitated. The bead complexes were then washed three times in 0.5 ml of lysis buffer before being resuspended in 20 μl of reducing sample buffer.

**Western Blot and Peptide Competition—**Proteins were separated by SDS-PAGE using 7.5% acrylamide/0.2% bisacrylamide gels and transferred to Immobilon P membranes. Proteins were detected on the western blot with the indicated primary antibodies diluted 1:1000 in PBS-1% bovine serum albumin and 0.05% sodium azide. All secondary antibodies were used at 1/10,000. Immunoreactive bands were visualized with the chemiluminescence Western blotting system (Amersham...
Biosciences). For peptide competition assays, the diluted primary antibodies were mixed 5 min prior using with 10 μg/ml of the indicated peptide.

ELISA—The ELISAs were performed using standard methods. Briefly, 10-amino acid peptides were coated onto ELISA plates (Nalge NUNC, Roskilde, Denmark) at 50 ng per well in pH 9 carbonate buffer. After blocking with PBS/0.05% Tween 20/5% FCS, the indicated dilution of PAP-1 was incubated for 1 h, and the bound immunoglobulins were assayed by a further incubation with a horseradish peroxidase-conjugated goat anti-rabbit antibody (DAKO Ltd., Ely, UK) and O-phenylene diamine tablets (Sigma-Aldrich, Gillingham, UK) as a colorimetric substrate. The values of the optical density measured in wells not incubated with PAP-1 but with diluent only, were subtracted.

Characterization of PAP-1 recognition pattern in primary human T cell lysates. A, T cells were left unstimulated (0) or were treated for 5 min with 100 μM pervanadate (PV). Total proteins from whole cell lysates were subjected to Western blot analysis using PAP-1. B, T cells were stimulated through their antigen receptor, or not (0), for 5 min with 10 μg/ml of the anti-CD3 cross-linking antibody UCHT1. Whole cell lysates were subjected to Western blot analysis with either PAP-1 (top panel), anti-P-Ser-256-FKHR (middle panel), mixed together with 10 μg/ml of the following competing oligopeptides. Lanes 1 and 2, non-phosphorylated sequence from GSK3α (RARTSSFAEP); lanes 3 and 4, P-Ser-256-GSK3α (RATSpSFAEP); lanes 5 and 6, P-Ser-256-FKHR (RRRAsPSMONN); lanes 7 and 8, P-Thr-23-IKKα (BerlinPTGGFG).Membranes were stripped and reprobed for GSK3. C, T cells were left non-stimulated (0) or treated with 10 μg/ml UCHT1 for 5 min in the presence of 1:1000 Me2SO (vehicle control). The indicated inhibitors were added 30 min before stimulation. Whole cell lysates were subjected to Western blot analysis with PAP-1 or anti-P-Ser473-PI3K and anti-P-ERK 1/2 antibodies. Membranes were stripped and reprobed with anti-pan GSK3α/β.

RESULTS

A polyclonal rabbit antiserum was raised against the phospho-peptide RARTSpSFAEP, where pS is a phospho-serine corresponding to the GSK3α Ser-21 sequence. In initial experiments to explore the reactivity of the resultant antiserum PAP-1, we carried out Western blot analyses of cell lysates isolated from quiescent and pervanadate (PV)-activated T lymphocytes. Pervanadate is used to pharmacologically induce a potent stimulation of tyrosine kinases and their downstream effecter pathways in lymphocytes and, hence, is a very useful tool for synchronously activating a broad range of serine kinases in these cells. PAP-1 recognized a single 160-kDa protein in the unstimulated T cells but was strongly reactive with a number of proteins across a wide molecular weight range isolated from pervanadate-activated cells (Fig. 1A). These have molecular masses of 50, 55, 75, 80, 85, 250, and 400 kDa, respectively. It is important to establish whether PAP-1 can recognize proteins phosphorylated in response to physiologically relevant stimuli, and, hence, the immunoreactivity of PAP-1 with T cell lysates isolated from antigen receptor activated T cells was examined. PAP-1 was strongly reactive with several proteins in cell lysates isolated from stimulated but not quiescent cells; the predominant proteins recognized by PAP-1 in TCR-activated cells were those with molecular masses of 50, 55, 85, and 250 kDa (Fig. 1B).
The phospho-peptide immunogen used to generate PAP-1 was based around Ser-21 in GSK3α. The p50 protein recognized by PAP-1 in activated T cells co-migrates on SDS-PAGE with GSK3α, and its identity as GSK3α was confirmed by its co-elution during protein purification on heparin-Sepharose and Mono Q columns (see below). The identity of the other proteins recognized by the antisera was unknown. Ser-21 in GSK3α is a substrate for PKB (27). However, the immunoreactivity of PAP-1 with these additional proteins was competed by the immunizing phospho-peptide phosphorylated PKB substrate peptide (28) or phospho-Thr-23 IKK (29). A commercial antiserum that recognizes phosphorylated FKHR serine 256 is available. This antiserum recognized a single protein of 97 kDa in quiescent T cells and two proteins of 85 and 97 kDa in antigen receptor-activated T cells (Fig. 2, middle panel). These did not co-migrate with any of the PAP-1-reactive proteins. Moreover, the immunoreactivity of the phospho-Ser-256 FKHR antisera was competed by the phospho-Ser-256 FKHR peptide but not the PAP-1-immunizing phosphoprotein-peptide or the phospho-Thr-23 IKKα peptide. The phospho-peptide competitions show that phospho-antisera are highly selective and indicate that PAP-1 reacts with proteins that contain a sequence structurally similar to GSK3α, Ser-21.

The TCR regulates GSK3α serine phosphorylation via PI3K-dependant pathways (9). The PI3K inhibitor LY294002 prevents the PKB activation response seen in TCR-activated cells. It also suppresses activation of the MAPKs Erk1 and -2 (Fig. 1C). TCR induction of the phosphorylation of GSK3α (PAP-1p50), PAP1 p55, p80/85, and p250 was inhibited by Ly294002. PAP-1-reactive proteins, like GSK3α, are regulated by PI3K pathways (Fig. 1C).

**PAP-1: A Phospho-peptide-Specific Antiserum That Recognizes a Subset of PKB Substrates**—A critical question was whether the PAP-1 antisera would be a pan PKB substrate antisera with the potential to identify novel substrates for this kinase. Moreover, an interesting question was whether the PAP-1 phospho-map shown in activated T cells was unique or seen in other cell lineages. To probe this issue we analyzed PAP-1 immunoreactivity with a human epithelial cell line, RPE, which expresses a tamoxifen-inducible PKB mutant. In quiescent RPE cells there is low basal activity of PKB and some basal phosphorylation of GSK3α on Ser-21 (see Fig. 4 below). PKB activity is induced by tamoxifen treatment of the cells with a concomitant increase in GSK3α Ser-21 phosphorylation (30).

The data (Fig. 2) show that PAP-1 recognizes two main proteins in quiescent RPE cells, a p50 protein and an unknown 200-kDa protein. In tamoxifen-activated RPE cells, PAP-1 recognizes two additional proteins of 65 and 120 kDa. These were not seen in RPE cells activated with phorbol esters that activate PKC but not PKB pathways. The immunoreactivity of PAP-1 was competed by the immunizing PAP-1 phospho-peptide. It is of note that the immunoreactivity pattern of PAP-1 with lysates from PKB-activated RPE cells was different from that seen in antigen receptor-activated T cells.

During the course of these experiments, Cell Signaling Technology (Beverley, MA) raised an antiserum against a mixture of phosphorylated PKB substrate peptides. This antiserum recognizes the PKB substrate sequence RXXRXpS/T, where X is any amino acid and is referred to as a CST-pan PKB phospho-substrate antisera (21, 22). An important question was whether PAP-1 is also a pan PKB substrate antisera with an overlapping immunoreactivity with this commercial antisera. Accordingly, the immunoreactivity of PAP-1 and the CST pan PKB phospho-substrate antisera on RPE cells that express a tamoxifen-inducible PKB was also compared (Fig. 2, A versus B). Key points were that the CST-pan PKB phospho-substrate antisera had a completely different pattern of reactivity to PAP-1 in RPE cells. The pan PKB substrate antisera did not react with a p50 protein corresponding to GSK3α. Nor did the pan PKB substrate antisera recognize the PAP-1-reactive p65 or p120 proteins; rather, it recognized a major protein, p70, in tamoxifen-activated cells and p70 plus an additional p97 protein in phorbol ester-activated cells. The pan PKB substrate antisera was also strongly reactive with a number of proteins migrating around p200 that were not seen with PAP-1. Strikingly, the pan PKB phospho-substrate antisera was strongly reactive with proteins from PDBu-activated RPE cells, whereas PAP-1 was not.

These results indicate that PAP-1 has the potential to recognize novel PKB substrates. However, its different pattern of immunoreactivity to the commercial pan PKB substrate antisera indicates that PAP-1 is not broadly reactive with all PKB substrates. One further indication that PAP-1 does not recognize all PKB substrates is given by the T cell data in Fig. 1. Thus both GSK3α and the faster migrating GSK3β are known PKB substrates, and commercial antisera selectively reactive with phospho-GSK3α and β thus recognize a doublet of proteins. In contrast, PAP-1 only detected the 50-kDa GSK3α protein and not the GSK3α/β doublet (Fig. 1, B and C).

To explore the selectivity of PAP-1 further, the immunoreactivity of this antisera was examined by ELISA. Fig. 3A shows that PAP-1 displayed a strong immunoreactivity with the immunizing phosphopeptide Ser-21 in GSK3α but did not recognize a range of other phosphorylated PKB substrate peptides, including phospho-Ser-256 in FKHR (28), phospho-Thr-32 in FKHLR1 (31), phospho-Thr-23 in IKK (29), and phospho-Ser-136 in BAD (32). To conclude, PAP-1 is not a pan PKB substrate antisera but rather a phospho-peptide specific antisera with the potential to recognize a subset of PKB substrates.

The previous data show that PAP-1 can recognize a subset of PKB substrates, but another question is whether it has a broader specificity. In this context, GSK3α Ser-21 phosphorylation can be regulated by PKB or via a PKC-mediated response.

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* S. Basu, unpublished.
CST-pan PKB antiserum (\(*\)). Membranes were stripped top right panel subjected to Western blot analysis using PAP-1 (\(*\)) or anti-P- or were treated with 50 ng/ml PDBu. Whole cell lysates were analyzed (\(*\)) recognizes phorbol ester-induced proteins. T cells were left non-stimulated (\(*\)) or were subjected to Western blot analysis using PAP-1 (\(*\)) top left panel top panel subjected to Western blot analysis with PAP-1 (\(*\)). This antiserum also recognizes proteins in phorbol ester-treated cells. The reactivity of CST-pan PKB antisera have different immunoreactivity and activated T cells. This band was not seen in PAP-1 immunoreactivity results are shown of PAP-1 with CST-pan PKB antiserum with antigen receptor or PDBu-activated T cells induce high levels of PAP1p50 (GSK3-\(\alpha\)–\(\beta\)) phosphorylation events at the level where there is convergence of signaling of multiple pathways. PAP-1 can therefore be used to pinpoint novel cell specific or stimulus specific phosphorylation events for future analysis.

Given the selectivity of PAP-1 for its cognate phospho-peptide, all of these proteins are likely to contain a phosphorylated peptide sequence structurally similar to GSK3\(\alpha\)a Ser-21. The next stage in this process was to purify PAP-1-identified proteins. From the experiments described above, the PAP-1-reactive protein p55 was selected for further work. The rationale for this choice was that this was an antigen receptor-regulated protein. Moreover, a comparison of the PAP-1 phospho-fingerprint of T cells and RPE cells indicated that p55 is selectively expressed/regulated in T lymphocytes. This was confirmed by extensive comparisons of the pattern of PAP-1 immunoreactivity with cell lysates isolated from different cell populations (data not shown).

Initial experiments found that the PAP-1 antiserum could not selectively immunoprecipitate proteins from activated T cells. Alternative experimental protocols were therefore devised to purify p55 to homogeneity for protein sequence analysis by mass spectrometry. Cell lysates were prepared from 9 \(\times\) 10\(^8\) activated T lymphoblasts, and the lysates were fractionated initially on heparin-Sepharose columns; a second step purification on Mono Q columns was performed followed by separation by two-dimensional SDS-PAGE gels. At each stage the presence of p55 in different column fractions was determined by Western blot analysis with PAP-1. Fig. 4 shows the elution profiles on heparin-Sepharose (Fig. 4A) and Mono Q columns (Fig. 4B) of p50 and p55 PAP-1-reactive proteins. p50 co-eluted with GSK3\(\alpha\), whereas p55 had distinct elution patterns. Fig. 4C (left panel) shows silver-stained two-dimensional SDS-PAGE gels of the final fractionation step; Fig. 4C (right panel) shows the corresponding PAP-1 Western blot analysis of the purified material. The protein spots corresponding to p55 were then digested with trypsin, and the tryptic peptides were analyzed by matrix-assisted laser desorption time-of-flight (MALDI-TOF) mass spectrometry (35). MALDI-TOF analysis identified a single oligopeptide from p55, and it proved impossible to obtain an effective fingerprint of this molecule. However, protein sequencing of p55 tryptic peptides identified that p55 contained the peptide (K\(\text{KFIYYDVD-LPEEA}GHRPSR\)) (Fig. 5A). This corresponded to amino acid residues 226–244 of a very recently described molecule known as SLY (Src homology 3 (SH3) domain-containing protein expressed in lymphocytes) (36). Ser-27 in SLY Is a Substrate for Antigen Receptor-activated Serine Kinases—To identify the phosphorylated residues that are recognized by PAP-1, we examined the sequences of SLY for peptides corresponding to the PAP-1-immunizing peptide. SLY comprises 380 amino acids, and Ser-27 had the closest homology to the GSK3-\(\alpha\)a Ser-21 peptide used to produce PAP-1 (Fig.
Accordingly, a phosphorylated peptide corresponding to SLY Ser-27 (LQRSSpSFK) was synthesized. PAP-1 was reactive with this phospho-peptide as judged by ELISA (data not shown). The ability of this peptide to compete PAP-1 immunoreactivity with p55 was also assessed. The results in Fig. 6A show that the phospho-peptide corresponding to Ser-27 in SLY was able to efficiently compete out PAP-1 immunoreactivity with p55.

No immunoprecipitating antiserum to SLY is available. Hence, to confirm SLY as a PAP-1-reactive protein, a Myc epitope-tagged SLY was expressed in A20 cells. These cells were then activated with pervanadate or via the B cell antigen receptor, and whole protein lysates were subjected to Western blot analysis with PAP-1 followed by reprobing with 9E10 anti-Myc to confirm expression. The data (Fig. 6B) show that SLY expressed in activated lymphocytes is immunoreactive with PAP-1, whereas SLY expressed in quiescent cells is not. These experiments also confirm that SLY is a substrate for antigen receptor-regulated serine kinases. To determine if Ser-27 in SLY is the sequence recognized by PAP-1, this residue was substituted with alanine. The SLY S27A mutant was also not reactive with PAP-1. These mutant studies thus establish Ser-27 in SLY as a substrate for antigen receptor-activated serine kinases.

The initial characterization of PAP-1 showed that this antiserum could recognize proteins phosphorylated via PKB or PKC pathways. In this context, the SLY Ser-27 sequence, LSLQRSSSFKDFKS, is not a good consensus PKB phosphorylation site. Instead, analysis of SLY with Scansite, a protein database motif-scanning program (19), indicated that Ser-27 is an optimum substrate sequence for protein kinase C family kinases. The data in Fig. 3 show that SLY phosphorylation was induced by phorbol ester activation of T cells. The data in Fig. 7A show also that SLY is not phosphorylated when T cells are activated by the cytokine interleukin 2. This cytokine is a strong activator of PKB and MAPKs and induces robust phosphorylation of GSK3b (Fig. 7A). However, IL-2 is unable to activate PKC, as judged by its inability to induce phosphorylation of a known downstream target of PKC, protein kinase D (10) (Fig. 7A). Furthermore, pretreatment of T cells with RO 31-8425, a broad specificity PKC inhibitor, prevents TCR-mediated phosphorylation of SLY without attenuating activation of PKB (Fig. 7B). These data collectively show that the regulated phosphorylation of SLY Ser-27 correlates with activation of PKC pathways in T cells.

**DISCUSSION**

Antisera broadly reactive with structurally related serine-phosphorylated peptides are emerging as valuable tools with which to unmask complex patterns of serine phosphorylation in cells. Serine phosphorylation is one of the most frequent post-translational modifications, but pathways of serine phosphorylation are difficult to resolve because it is difficult to distinguish basal serine phosphorylations from extracellular signal-regulated events. The challenge has been how to resolve the huge background noise seen in most forms of analysis. The present data describe a novel antiserum PAP-1 that can be used for this purpose. The rationale behind the generation of absorbance at 280 nm is shown by the full line, and the elution NaCl gradient is shown by the broken line. Aliquots of 4 μg of protein from each fraction were prepared as above and Western blotted with PAP-1. C, the Mono Q fractions rich in PAP1p55 were desalted, acetone-precipitated, and run in duplicate using two-dimensional SDS-PAGE. From each duplicate, one gel was treated for total protein content by silver staining (right panels), whereas the other was subjected to Western blotting with PAP-1 (left panels).
PAP-1 was that phospho-peptide-specific antisera would cross-react with proteins that share similar phosphorylated peptide sequences. The cellular system used to exploit this strategy was antigen receptor-activated human T lymphocytes. One evolutionary conserved pathway in T cells is the regulated phosphorylation of the serine kinase GSK3 on Ser-21. The present results show that an antiserum termed PAP-1, which was raised against a phospho-peptide RARTS\(^{\text{pS}}\)FAEP, corresponding to GSK3\(^{\text{Ser-21}}\), can be used to define other substrates for antigen receptor-regulated kinases that are phosphorylated in parallel to GSK3\(^{\text{Ser-21}}\) and contain a sequence structurally similar to GSK3\(^{\text{Ser-21}}\). PAP-1 thus recognizes a tier of serine kinase substrates regulated by antigen receptors via PI3K-mediated pathways.

There have been recent descriptions of pan-kinase substrate antibodies, i.e. antibodies that can be used to identify substrates for known serine kinases such as PKB (22, 23). The immunizing peptide for PAP-1 is a PKB substrate, but it should be emphasized that PAP-1 only recognizes a subset of PKB substrates. Moreover, the PAP-1-immunoreactive pattern in activated T cells and epithelial cells is very different from the immunoreactivity pattern of the recently characterized Cell Signaling Technology "pan PKB substrate antisera" (CST-pan PKB) raised against the panel of peptides with the consensus R\(^{\text{XXX}}\)pS/T, where X is any amino acid. It should also be emphasized that neither PAP-1 nor CST-pan PKB antisera have immunoreactivity restricted to PKB substrates. Both recognize proteins phosphorylated via PKC pathways in T lymphocytes. Moreover, the CST-pan PKB antisera recognize proteins phosphorylated in response to PKC activation in epithelial cells, whereas PAP-1 does not. These antibodies are thus very useful for phospho-proteomic analysis of cell activation, but they are not useful as reporters for activation of a particular kinase pathway.

The PAP-1 antiserum had a very distinct pattern of immunoreactivity in different cell types. From simple comparative analysis, it was possible to identify proteins that were regulated by antigen receptors and apparently unique to T cells. Hence, in activated epithelial cells PAP-1 recognized GSK3\(^{\text{Ser-21}}\) and proteins of 65 and 120 kDa, whereas in activated T cells PAP-1 predominantly recognized GSK3\(^{\text{Ser-21}}\) and p55 and p85. Analysis with PAP-1 thus could pinpoint novel targets in pathways of antigen receptor-regulated serine phosphorylation. Similarly, in epithelial cells expressing an inducible active PKB, PAP-1 could identify novel proteins phosphorylated by a PKB pathway. Interestingly, the PAP-1-reactive proteins in T cell or epithelial cells were not recognized by the CST pan PKB substrate antisera. CST pan PKB is broadly reactive with a range of phospho-peptides that are substrates for the ACG family of serine kinases, including PKB/Akt, but the present data show it does not recognize all substrates for this kinase. PAP-1 and CST-pan PKB thus appear to recognize non-overlapping subsets of PKB substrates. Accordingly, it looks like it will be difficult to get a complete analysis of protein phosphorylation pathways using a single antisera broadly reactive with serine-phosphorylated peptides. Instead, a combination of dif-

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**Fig. 5. Identification of PAP1p55 using mass spectrometry.** 400 µg of fractions enriched for p55 was separated by two-dimensional SDS-PAGE, and the proteins were visualized by colloidal Coomassie Blue stain (not shown). A, tryptic digests of PAP1p55 were analyzed using nano-LC coupled to a Q-TOF analyzer. The sequence of an identified 19-amino acid fragment was sequenced and matched to residues 226–244 of SLY and is shown beneath the digest pattern. B, schematic and domain organization of SLY. The sequence representing the most likely site of recognition for PAP-1 and the potential phosphorylation site are indicated.
ferent antisera that are selective against different peptides will be required.

One of the PAP-1-reactive proteins in antigen receptor-activated T cells, p55, was selected for further characterization because it was regulated by antigen receptors and apparently unique to T cells. p55 was then purified and identified as SLY, the prototypical member of a recently identified family of adapter proteins with restricted expression in hematopoietic cells (36). The analytical work with PAP-1 had pinpointed p55 as a lymphoid-restricted protein, and SLY indeed has restricted expression to lymphoid tissues. SLY was originally found "accidentally" as part of an expression screen for genes that could stimulate adhesion of T lymphoma cells to endothelial cells (36). The SLY gene product was not able to induce cell adhesion, but its isolation was reported because its structure suggested that it might function as a signaling adapter protein. Northern blot and in situ hybridization analysis showed a preferential expression in lymphoid tissues, and the SLY gene is located on the X chromosome in proximity to genes involved in various immune disorders. SLY was so named because it is an Src homology 3 (SH3) domain-containing protein expressed in lymphocytes. The protein also contains a sterile motif (SAM). SH3 domains are comprised of about 50 amino acid residues that bind to proline-rich sequences of proteins with the consensus PXXP. SAM domains mediate homodimerization and heterodimerization of proteins (37). This structure makes it very likely that SLY can act as an adapter, although there have been no studies of its interaction partners. Interestingly, other hematopoietic proteins closely related to SLY have been described recently, HACS1 (38) and NASH (39). SLY, HACS1, and NASH have a similar organization of SH3 and SAM domains and appear to be restricted in expression to hematopoietic cells. Serial analysis of gene expression identified NASH as a gene preferentially expressed in mast cells; HACS1 was found in a wide range of hematopoietic cells, whereas SLY is preferentially expressed in lymphoid cells. The structures and restricted tissue expression of SLY, HACS1, and NASH suggest that these proteins might have a specific role in hematopoietic cells. The present data provide the first insights into the position of these adapter proteins in the context of signal trans-
duction in lymphocytes by demonstrating that Ser-27 in SLY is a physiological substrate for antigen receptor-activated serine kinases. Moreover, SLY phosphorylation seems to occur selectively in response to antigen receptor engagement via a PKC-mediated pathway that cannot be triggered by cytokines. Ser-27 in SLY is contained within the sequence LQRSSSFK, and this sequence is conserved in SLY in different species from fish to mammals. Moreover, it is striking that the serine at position 27 and the surrounding sequence (RSSFK) is conserved in the SLY-related proteins HACS1 and NASH. The conservation between SLY, HACS1, and NASH in terms of the PAP-1-recognized phospho-peptide region provides clear biological insight into where to position other members of this adapter protein family in signal transduction cascades in hematopoietic cells. The identification of SLY as an antigen receptor-regulated serine kinase target also shows that the phospho-proteomic approach with PAP-1 described herein can identify links between antigen receptors and novel molecules and give some insight as to when and where such proteins might be important in T cells.

In conclusion, the present study describes an antisera raised against a defined phospho-peptide that can be used as a tool for phospho-proteomic analysis of cells. Herein it has been used to probe lymphocyte activation. PAP-1 and various commercial phospho-peptide-specific antisera have unique patterns of immunoreactivity with activated T cell lysates and can be used to make a phospho-map of TCR-activated cells. Phospho-specific antisera thus opens a window that can reveal the existence of previously unknown targets for antigen receptor signal transduction as well as a new dimension for signal transduction studies in lymphocytes.

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J. Biol. Chem. 2003, 278:9267-9275.
doi: 10.1074/jbc.M211252200 originally published online January 5, 2003

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