Review

Phosphorylation State-Specific Antibodies

Applications in Investigative and Diagnostic Pathology

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Until recently, the investigation of protein phosphorylation was limited to biochemical studies of enzyme activities in homogenized tissues. The availability of hundreds of phosphorylation state-specific antibodies (PSSAs) now makes possible the study of protein phosphorylation *in situ*, and is opening many exciting opportunities in investigative and diagnostic pathology. This review illustrates the power of PSSAs, especially in immunohistochemical applications to human disease and animal models. Technical considerations, including antibody specificity and lability of phospho-epitopes, are covered, along with potential pitfalls, illustrated by a case study. In the arena of oncology, PSSAs may prove especially valuable in directly demonstrating the efficacy of chemotherapies targeted at protein kinase cascades. Novel applications of PSSAs are also beginning to reveal molecular mechanisms of inflammatory, degenerative, and toxin-induced diseases. (Am J Pathol 2003, 163:1687–1698)

Protein phosphorylation was discovered more than 50 years ago.¹ The study of intracellular signaling is nearly synonymous with the study of protein phosphorylation. In a recent review, Philip Cohen² asserted that “the reversible phosphorylation of proteins regulates nearly every aspect of cell life.” Phosphorylation and its reverse reaction, dephosphorylation, are carried out by more than 2000 kinases and a smaller number of phosphatases, respectively. Phosphorylation of proteins can modify function in two main ways. Phosphorylation at specific residues may induce conformational changes that lead to altered enzymatic activity, or of ion channel permeability. Alternatively or in parallel, phosphorylation can generate specific binding sites for other proteins, leading to the assembly of functional complexes of proteins. Phosphorylation of transcription factors is central to gene regula-

tion whereas phosphorylation of cytoskeletal proteins is important for control of cell shape and motility. Finally, recent advances point to phosphorylation as an initial step in targeted protein degradation.³ The importance of protein phosphorylation in cancer was made apparent by the identification of several oncogenes as mutated and constitutively activated kinases.⁴ Malfunction of signal transduction pathways is also implicated in non-neoplastic diseases including immunological, endocrine, cardiovascular, cerebrovascular, and neurodegenerative disorders.

The development of phosphorylation state-specific antibodies (PSSAs) now makes possible the study of protein phosphorylation *in situ*, allowing glimpses of dynamic protein phosphorylation reactions in the spatially complex structures of cells and tissues. I will review the historical development of PSSAs, briefly describe current methodologies for their production, and address potential pitfalls and artifacts, along with a case study from our laboratory. I will then present selected applications of PSSAs, limiting the review to studies on human disease or animal models and including immunohistochemical approaches.

Development of PSSAs

The first PSSAs were monoclonal antibodies raised against neurofilament proteins. Their recognition as PSSAs was a serendipitous discovery followed by rigorous analysis.⁵ In this elegant landmark paper, the authors showed not only that PSSAs could discern between phosphorylated and dephosphorylated proteins in biochemical assays, but that they could be applied to fixed brain tissue sections to reveal information about the subcellular heterogeneity of neurofilament protein phosphor-

Supported by a National Institutes of Health Independent Scientist Award (K02 NS02065) and an American Cancer Society institutional research grant.

Accepted for publication July 17, 2003.

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ylation. The panel of Sternberger antibodies remains a mainstay in diagnostic and investigative neuropathology.

In the 1980s, researchers succeeded in developing monoclonal and polyclonal antibodies specific for phosphotyrosine, and with somewhat more limited success to phosphothreonine.^{6–8} These antibodies, because they bind to any protein containing the phosphorylated amino acid, were most useful in Western blotting experiments, where the multiple phosphoproteins could be resolved by their molecular weight. Nevertheless, investigators have applied these generic PSSAs in immunohistochemical studies of human cancers and revealed elevated accumulation of phosphotyrosyl-containing proteins in cancers.⁹ Anti-phosphotyrosine antibodies were instrumental in defining the signaling protein complex localized to focal adhesions.¹⁰ More recently, a phosphotyrosine antibody was successfully used to screen anaplastic lymphoma specimens for evidence of activating ALK tyrosine kinase mutations.¹¹ This could be a general approach for cancer geneticists who wish to identify subsets of tumors likely to harbor mutationally activated tyrosine kinases.

More targeted approaches to develop sequence-specific PSSAs yielded success in the early 1990s. Again focusing on the cytoskeleton, Inagaki and colleagues¹² produced monoclonal antibodies specific for phosphorylated forms of glial fibrillary acidic protein (GFAP) by injecting mice with enzymatically phosphorylated peptides, and screening resultant hybridomas for reactivity against the phospho-, but not the dephospho-peptide. The phosphopeptide immunization approach was also taken by the Greengard group,¹³ but with the production of polyclonal antisera instead of monoclonal antibodies. Polyclonal PSSA production is less labor intensive, in that the extensive post-immunization screening step is omitted, but does require an affinity purification step. The most effective procedure is to first remove those antibodies in the antisera that bind to the dephosphorylated antigen by using a dephospho-peptide affinity column, followed by positive selection of the flow-through on a phospho-peptide column. It should be noted that it is just as feasible to produce dephospho-specific antibodies (those that bind to a specific site only when dephosphorylated). To do so, animals are immunized with the appropriate dephospho-peptide, and the resultant antisera are affinity purified to remove antibodies reactive to the phosphopeptide. Although early methods often relied on enzymatic phosphorylation of peptide antigens, a very inefficient process, advances in peptide chemistry allowing chemical phosphorylation have largely obviated the inefficient enzymatic phosphorylation procedures.¹³ The first PSSAs developed using synthetic tyrosine-containing phosphopeptides were used to study activation of erbB-2.^{14,15}

Commercially available PSSAs now are available for more than 300 different phosphoproteins and/or phosphorylation sites (Table 1; obtained by metasearch of catalogs using <http://www.biocompare.com>). The majority of commercial PSSAs are produced by immunization of animals (usually rabbits or goats) with a synthetic phosphorylated peptide. In theory, it would be possible to

generate a PSSA against every possible phosphopeptide by synthesizing all possible phosphopeptides. Even if the peptide length is limited to 11 amino acids, the number of peptides needed for complete coverage of all sequences in the form XXXXXA_pXXXXX, where X is any amino acid and A_p is either phosphothreonine, phosphoserine, or phosphotyrosine is 3×10^{20} . Unfortunately, this would require unachievable manpower, not to mention a rabbit population covering the face of the earth. More realistic approaches might limit the universe of phosphopeptides to those that actually occur in nature, perhaps on the order of tens of thousands, assuming a handful of sites per phosphoprotein. In fact, large-scale identification of phosphorylation sites is now possible using mass spectrometric techniques, and this method has the potential to eventually map the entire phosphoproteome (reviewed in ¹⁶).

Tests of Antibody Specificity

The principles of testing for PSSA specificity are generally similar to conventional antibodies. The most important additional controls involve experimentally altering the phosphorylation state of the target protein and demonstrating the ability of the PSSA to document this alteration in fixed cells or tissues. An example of a rigorously controlled immunohistochemical study using a monoclonal PSSA was recently published.¹⁷ The authors used a phospho-Stat5 antibody to examine phosphorylation in a culture model as well as mouse and human breast tissue specimens. Several independent methods were used to validate antibody specificity, including Western blotting, immunostaining of stimulated *versus* unstimulated cultured cells, immunostaining of tissue with peptide preincubation controls, and genetic (knockout) controls. Each of these approaches is discussed briefly below.

Western Blotting

As for any antibody, the first step is validation by Western blot to show that it detects a single band (or multiple bands if family members share phosphorylation motifs) of appropriate molecular weight in homogenates of tissues and cells. The antibody should also report phosphorylation and dephosphorylation in response to appropriate stimuli in cell culture models.

Immunocytochemical Staining of Fixed Cultured Cells

The next step is to show that the phosphorylation state changes observable on Western blots are also observable on immunostaining of fixed cultured cells undergoing the same stimulation paradigm. The fixation should mirror that which takes place in the surgical pathology lab (eg, typically 10% buffered formalin). Additional assurance that the PSSA in hand will work on formalin-fixed, paraffin-embedded (FFPE) sections can be had by demonstration that fixed cultured cell preps, after paraffin-embedding and sectioning, still retain the expected

changes in immunoreactivity. Methods for preparation of cultured cells for paraffin-embedding have been compared for the purpose of cell and tissue microarray construction.¹⁸

Phosphopeptide Preincubation and Enzymatic Dephosphorylation Controls

A common test of specificity for antibodies in general is preincubation of the diluted antibody with molar excess of the immunizing antigen. The same can be done for PSSAs, with additional specificity provided by the use of phospho- and dephosphopeptides. Immunizing phosphopeptides are often available from antibody suppliers. An important control is to show that the phosphopeptide, but not the otherwise identical dephosphopeptide, abolishes immunoreactivity (see¹⁷ and¹⁹ for examples). A less specific but still useful control is to show that immunoreactivity for a phospho-dependent antibody is abolished by pretreatment of sections with alkaline phosphatase (see¹⁹ for technical details). In contrast, dephospho-specific antibodies, for example tau-1, which only binds to a specific dephosphorylated epitope, should show enhanced immunoreactivity after alkaline phosphatase treatment.²⁰

Molecular Genetic Controls

An elegant control at the molecular level is to perform site-directed mutagenesis of the phospho-acceptor amino acid within the protein of interest. Expression of the mutated protein in cells, followed by immunoprecipitation and probing with the PSSA should reveal complete loss of immunoreactivity compared to the wild-type protein. This approach has been taken for a phosphorylation-specific phospholipase C- γ 2 antibody²¹ and the phospho-specific Stat5 antibody.¹⁷

Immunohistochemical Artifacts Specific to PSSAs

As with immunohistochemistry in general, the most common problem encountered in the application of PSSAs to fixed tissue sections is a false-negative reaction. Tissues that are known to contain the phosphorylated protein of interest, as determined by Western blot with the PSSA, sometimes fail to exhibit immunoreactivity by immunohistochemistry. This can be due to inaccessibility of antigen to antibody, "antigen masking," or sensitivity (signal-to-noise ratio). Antigen retrieval techniques may improve detection of phosphoepitopes, especially within dense cellular matrices, such as the nucleus. Systematic testing of various antigen retrieval techniques for use with PSSAs has not been reported. The problem of sensitivity is especially apparent for some phosphorylated signaling proteins and transcription factors which are present at low copy number and may not be detectable by standard immunohistochemical methods. An obvious warning sign that a PSSA is probably not suitable for immunohisto-

chemical applications is when the manufacturer recommends a two-step procedure (immunoprecipitation followed by Western blotting) for phosphorylation detection on blots. This generally indicates a relatively low affinity and/or selectivity of the PSSA.

A key concept to keep in mind when using PSSAs is that protein phosphorylation is a highly dynamic process *in vivo*. At best, immunohistochemical detection of phosphorylation captures a true snapshot of phosphorylation at a moment in time. At worst, the snapshot captures only a fleeting trace of reality. In fact, there is potential for both under-representation and over-representation of the true phosphorylation state. A good case example is PSSAs directed against tau, the microtubule-associated protein that accumulates in Alzheimer's disease (AD) neurofibrillary tangles. A widely accepted notion in the field was that tau deposited in AD tangles had qualitatively and quantitatively aberrant phosphorylation. PSSAs were developed that detected hyperphosphorylated tau in AD but not normal brain.²² However, the subsequent discovery that biopsy-derived tau from normal non-AD brain (rapidly procured surgical tissue) was phosphorylated at many of the sites considered AD-specific shook up the field.²³ From this study the authors concluded that the phosphorylation sites under study were not AD-specific, but that AD neurons appeared to have deficient phosphatase activity relative to normal brain neurons, such that the phosphoepitopes disappeared from normal neurons by the time of autopsy, whereas AD neurons retained high levels of phospho-tau. To make matters more complicated, a comprehensive study of simulated post-mortem conditions revealed that tau initially underwent increased phosphorylation on at least one AD site, followed by a slow decrease.²⁴ Moreover, total protein phosphorylation underwent changes following tissue removal, with peaks of phosphorylation at 30 and 90 minutes postmortem, followed by a slow decline. The mechanism of these changes occurring in devitalized tissue is not completely understood. The obvious explanation for the slow decline is the loss of cellular ATP, required for kinase activity, in the face of continued phosphatase action (ATP-independent). However, the biphasic increases in total phosphorylation remain to be explained.

We have observed a related artifact in gathering data for a study on ERK/MAP kinase activation in human gliomas.¹⁹ In rapidly procured but large (>1 cm) surgical specimens, phospho-ERK/MAPK immunoreactivity was sometimes limited to the outer few millimeters of tissue compared to the uniform preservation of total (phospho-independent) ERK/MAPK immunoreactivity (Figure 1). This was observed in both low- and high-grade gliomas. Our interpretation of this observation is that due to time-dependent penetration of the formalin fixative, the outer rim of large specimens shows preservation of the phosphorylation state, whereas tissue deep in the core has lost phosphorylation by the time that the fixative has permeated. For this reason, we limit our analysis of PSSA immunoreactivity to either small biopsy specimens (<5 mm) or to the outer few millimeters of large specimens.

These observations, taken together with the clearly documented lability of phospho-tau epitopes, raise the

Table 1. Commercially Available Phosphorylation State-Specific Antibodies

4E-BP1 (S65)	eEF2 (T56)	Keratin 8 (S73)
4E-BP1 (T37/46)	eEF2k (S366)	LAT (Y171)
4E-BP1 (T70)	EGF Receptor (Y1045)	LAT (Y191)
Acetyl-CoA Carboxylase (S79)	EGF Receptor (Y1068)	LAT (Y226)
Adducin (S724)	EGF Receptor (Y845)	Lck (Y505)
AFX (S193)	EGF Receptor (Y992)	Leptin Receptor (Y985)
Akt (S473)	eIF2 alpha (S51)	Leptin Receptor (Y1138)
Akt (T308)	eIF4 G (S1108)	Lyn (Y507)
ALK (Y1604)	eIF4E (S209)	M-CSF Receptor (Y723)
AMPK-alpha (T172)	Elk-1 (S383)	M-CSF Receptor (Y809)
APP (T668)	eNOS (S1177)	MAPKAPK-2 (T222)
ASK1 (S83)	eNOS (T495)	MAPKAPK-2 (T334)
ATF-2 (T 69/71)	eNOS (S116)	MARCKS (S152/156)
ATF-2 (T71)	EphA3 (Y596/Y602)	MDM2 (S166)
Aurora 2 (T288)	Ephrin B (Y324/329)	MEK1 (T292)
Bad (S112/136)	ERK1/2 (T202/Y204)	MEK1 (S298)
Bad (S136)	Erk5 (T218/Y220)	MEK1/2 (S 217/221)
Bad (S155)	Estrogen Receptor alpha (S104/106)	Met (Y1234/1235)
Bcl-2 (S70)	Estrogen Receptor alpha (S118)	Met (Y1349)
Bcr (Y177)	Estrogen Receptor alpha (S167)	MKK3/MKK6 (S 189/07)
beta-Arrestin 1 (S412)	Ezrin (T567)/Radixin (T564)/Moesin (T558)	Mnk1 (T 197/02)
beta-Catenin (S33/37/T41)	Etk (Y40)	MSK1 (S360)
beta-Catenin (T41/S45)	FADD (S191)	MSK1 (S376)
BLNK (Y96)	FADD (S194)	MSK1 (T581)
BRCA1 (S1189)	FAK (Y397)	mTOR (S2448)
BRCA1 (S1280)	FAK (Y576/577)	mTOR (S2481)
BRCA1 (S1387)	FAK (Y925)	Myelin Basic Protein
BRCA1 (S1423)	FGF Receptor (Y653/654)	Myosin Light Chain 2 (S19)
BRCA1 (S1457)	FKHR (T24)	MYPT1 (T696)
BRCA1(S466)	FKHR (S256)	Neurofilament H,M,L
Btk (S180)	FKHRL1 (S 253)	Neurogranin (S36)
Btk (Y223)	FKHRL1 (T 32) P	NF-kappa B p65 (S536)
C/EBP beta (S105)	FLT3 (Y591)	Nibrin/nbs1 (S343)
c-Abl (T735)	FLT3 (Y591)	NMDAR1 (S890)
c-Abl (Y245)	FRS2-alpha (Y436)	NMDAR1 (S896)
c-Cbl (Y731)	Gab1 (Y627)	NMDAR1 (S897)
c-Cbl (Y774)	GABA-B ReceptorR2 (S892)	nNOS (S1416)
c-Jun (S63) II	GFAP	NR1 (S896)
c-Jun (S73)	GluR1 (S831)	NR2B (S1303)
c-Jun (S73)	GluR1 (S845)	NPM (T199)
c-Kit (Y719)	GluR2 (S880)	p38 MAP Kinase (T180/Y182)
c-Kit (Y703)	Glycogen Synthase (S640)	p53 (S15)
c-Kit (Y936)	GRF1 (S916)	p53 (S20)
c-Myc (T58/S62)	GSK-3 alpha/beta (S21/9)	p53 (S37)
Caldesmon (S789)	GSK-3 alpha (S21)	p53 (S392)
CaMKII (T286)	GSK-3 beta (S9)	p53 (S46)
Caveolin-1 (Y14)	GSK3 (Y279/Y216)	p53 (S6)
CD19 (Y531)	HER2/ErbB2 (Y1112)	p53 (S9)
cdc2 (T161)	HER2/ErbB2 (Y1248)	p70 S6 Kinase (T389)
cdc25 C (T48)	HER2/ErbB2 (Y877)	p70 S6 Kinase (T421/S424)
cdc25C (S216)	Histone H3 (S10)	p70 S6 Kinase (T 412)
cdk2 (T160)	Histone H2A.X (S139)	p90RSK (S380)
Chk1 (S296)	Histone H2B (S14)	p90RSK (T359/S363)
Chk1 (S317)	H3 (S28)	p90RSK (T573)
Chk1 (S345)	Histone H3 (T3)	p95/NBS1 (S343)
Chk2 (S19)	HMGN1/HMG-14 (S6)	PAK2 (S20)
Chk2 (S33/35)	HSP27 (S15)	Paxillin (Y118)
Chk2 (T387)	HSP27 (S78)	PDGF Receptor beta (Y751)
Chk2 (T432)	HSP27 (S82)	PDGF Receptor beta (Y716)
Chk2 (T68)	IGF-IR (Y1131)/Insulin Receptor (Y1146)	PDK1 (S241)
Cofilin (S3)	IKK alpha/beta (S180)	PDK1 (Y373/376)
Connexin 43 (S368)	I kappa B-alpha (S32/36)	PERK (T980)
CPI-17 (T38)	IRS-1 (S307)	Phospholamban (S16)
cPLA2 (S505)	IRS-1 (S612)	SAPK/JNK (T183/Y185)
CREB (S133)	IRS-1 (S636/639)	PAK1 (S144)/PAK2 (S141)
CrkII (Y221)	Jak1 (Y1022/1023)	PAK1/2 (S199/204)
CrkL (Y207)	Jak2 (Y1007/1008)	PAK1 (T423)/PAK2 (T402)
DARPP-32 (T34)	JNK/SAPK (T183/Y185)	PRK1 (T778)/PRK2 (T816)
DARPP-32 (T75)	KDR (Y1212)	PKA RII (S96)
delta-Opioid Receptor (S363)	Keratin 18 (S33)	PKC alpha (S657)
DNA-topoisomerase II alpha	Keratin 8 (S431)	PKC alpha/beta II (T638/641)

(Table continues)

Table 1. *Continued*

PKC delta/theta (S643/676)
PKC delta (T505)
PKC Epsilon (S 729)
PKC theta (T538)
PKC zeta/lambda (T410/403)
PKD/PKC mu (S 744/748)
PKD/PKC mu (S916)
PKD2 (S876)
PKR (T446/451)
PLC beta 3 (S1105)
PLC beta 3 (S537)
PLC gamma1 (Y783)
PP1 alpha (T320)
PP2A (Y307)
Presenilin-2 (S327/330)
Progesterone Receptor (S190)
Progesterone Receptor (S294)
PTEN (S380/T382/383)
Pyk2 (Y402)
Rac1/cdc42 (S71)
Rad17 (S645)
Raf (S259)
Raf (S338)
Rb (S780)
Rb (S795)
Rb (S807/811)
Ret (Y905)
RNA polymerase II
RSK3 (T 353/356)
S6 Ribosomal Protein (S235/236)
S6 Ribosomal Protein (S 240/244)
SEK1/MKK4 (T261)
SGK
Shc (Y239/240)
Shc (Y317)
SHP-2 (Y542)
SHP-2 (Y580)
Smad1/5 (S 463/465)
Smad2 (S465/467)
Src (Y416)
Src (Y527)
Stat1 (Y701)
STAT1 (S727)
STAT2 (Y689)
Stat3 (S727)
Stat3 (Y705)
Stat5 (Y694)
STAT5A/B (S726/S731)
Stat6 (Y641)
Syk (Y323)
Syk (Y525/526)
Synapsin (S9)
Tau (S199/S202/T205)
Tau (T212/S214)
Tau (S231)
Tau (S396/S404)
Tau (dephospho S199/S202)
TrkA (Y490)
Tuberin (T1462)
Tuberin (Y1571)
Tyk2 (Y 1054/1055)
Tyrosine Hydroxylase (S40)
VASP (S157)
VASP (S239)
VEGF Receptor-2 (Y951)
VEGF Receptor-2 (Y996)
Zap-70 (Y319)
Zap-70 (Y493)

Figure 1. Illustration of potential pitfalls and unexpected spatiotemporal complexity of protein phosphorylation using PSSAs. **A–D:** Artifactual loss of phospho-ERK immunoreactivity in large tissue specimens. Glioma specimens larger than 1 cm sometimes showed strong phospho-ERK staining in the peripheral few millimeters but not the core (**A**, anaplastic astrocytoma; **C**, glioblastoma multiforme). Immunoreactivity for a phosphorylation-independent ERK antibody was always preserved throughout the tumor specimen (**B** and **D**), suggesting that phosphorylated epitopes may be lost due to ongoing dephosphorylation in the core of large specimens due to slow fixation. **Bar** in **A**, 1 cm, applies to **A–D**. **E–F:** Comparison of phospho-MEK and phospho-ERK immunohistochemistry reveals unexpected complexity of component activation within a signaling cascade. A phospho-MEK antibody (Cell Signaling Technology) shows strong and selective staining of mitotic cells in a human glioblastoma (**E**, arrows). In contrast, phospho-ERK immunostaining of an adjacent section revealed cytoplasmic and nuclear staining in non-mitotic cells (**F**). These patterns were confirmed in several non-neoplastic tissues, in both human and rodent (J.W. Mandell, unpublished data). Antibody specificity was confirmed by both Western blot and peptide competition controls (data not shown). The apparent paradox was recently revealed to be due to a mitosis-specific cleavage of MEK which renders it highly phosphorylated in mitosis, but uncoupled to ERK activation.²⁷ **Bar** in **E**, 50 μ m, applies to **E–F**.

issue of phosphoepitope lability as a critical one in the field. Once fixed and paraffin-embedded, it seems likely that phosphoepitopes, like other antigens, are extremely stable in room temperature archives. However, this should be examined in an objective fashion, by comparing results from specimens over a several decade span of storage. Most important is the lability of protein phosphorylation in surgical specimens, both during the time of specimen handling and during fixation. There is a need for a controlled study of the dephosphorylation kinetics of representative phosphoepitopes in various human tissue specimens. This could be assessed both by quantitative Western blot as well as semi-quantitative immunohistochemistry. Clearly, it will not be possible to control the exact handling time for all surgical specimens, with variable handling times necessitated by frozen section diagnosis. Nevertheless, knowing the half-life of phosphorylation will give more confidence in interpreting results using PSSAs.

Table 2. Some Published Studies of Human Neoplasms Utilizing Phosphorylation State-Specific Antibodies

Phosphoprotein	Neoplasms	References
Transmembrane receptor tyrosine kinases		
ErbB-2	Breast carcinoma	28–32
EGFR	Non-small cell lung carcinoma (NSCLC), pancreatic carcinoma, testicular erm cell tumors	33–35, 62
IGF-1R	Breast carcinoma, medulloblastoma	36–38
CSF-1R	Breast carcinoma	39
Intracellular kinases		
ERK/MAP kinase	Gliomas, carcinomas (prostate, breast, pancreatic, head/neck)	19, 40–42, 62
AKT	Carcinomas (epithelial ovarian, endometrial, breast, papillary thyroid, pancreatic, NSCLC), multiple myeloma	43–48, 62
Transcription factors/nuclear proteins		
STAT3/6	Hodgkin lymphoma, multiple myeloma, prostate carcinoma	49–52
Beta-catenin	Colorectal carcinoma	53
Smad2/4	Breast carcinoma	54
I-kappaB alpha	Gynecologic squamous intraepithelial neoplasia/carcinoma	55
P53	Transitional cell carcinoma	56
Rb	Uveal melanoma, retinoblastoma	57, 58
Histone H1	Gynecologic squamous intraepithelial neoplasia/carcinoma	59
c-jun	Breast carcinoma, astrocytoma	60,61

Optimal fixation conditions for PSSAs have not been rigorously addressed. Data presented in a commercial publication (*Signals* 1:4, 2002; Cell Signaling Technologies) suggested that neutral-buffered formalin was the best all-around fixative for a panel of four different PSSAs, compared to 70% ethanol and paraformaldehyde: good news for studies on archival pathology specimens.

Taken together, these studies raise possible objections to using autopsy-procured tissues for studies with PSSAs. Nevertheless, PSSAs to tau, in particular, have proven powerful tools in the investigation and diagnosis of AD and other neurodegenerative diseases. Phosphorylated proteins that accumulate in aggregates or inclusions may be much more resistant to postmortem dephosphorylation than normally localized phosphoproteins.

Interpretation of Unexpected Staining Patterns: Potential Pitfalls or Opportunity for Discovery

Investigators using PSSAs in novel tissues or tumor types will occasionally reveal unexpected staining patterns. This can represent an opportunity for discovery but can also result from aberrant antibody cross-reaction. One approach to such an unexpected result from our laboratory is illustrated (Figure 1). We had used a commercially available phospho-MEK antibody (Cell Signaling Technology, Beverly, MA) to complement a phospho-ERK antibody study of Ras→Raf→MEK→ERK pathway activation in human gliomas. Since ERK is the only known substrate of MEK, and all culture models suggested simultaneous activation of the two components, it was expected that the immunostaining pattern of the phospho-MEK antibody would be identical to that for phospho-ERK. To our surprise, the phospho-MEK antibody labeled almost exclusively mitotic cells. This was true in both neoplastic gliomas (Figure 1E) and all non-neoplastic tissues examined, such as developing brain (not

shown). The staining pattern of the phospho-MEK antibody was identical to a well-characterized mitosis-specific monoclonal antibody, MPM-2 (data not shown).²⁵ The same tissues stained with two different phospho-ERK antibodies (Sigma, St. Louis, MO) showed cytoplasmic and nuclear immunoreactivity that was largely excluded from mitotic cells (Figure 1F).¹⁹ Activated and phosphorylated ERK has been reported to localize at the kinetochores of mitotic cells.²⁶ Thus it seemed possible that there might be a role for phosphorylated and activated MEK in mitosis. Nevertheless, the largely discordant staining patterns of phospho-ERK and phospho-MEK were confusing and raised the question of aberrant antibody cross-reactivity. Resolution of the paradoxical difference in phospho-ERK and phospho-MEK immunoreactivity came from a series of biochemical experiments (J.W. Mandell, M. Zecevic, and M.J. Weber, unpublished data) and was also confirmed by a recent publication.²⁷ MEK1 (but not MEK2) appears to undergo a mitosis-specific cleavage, dependent on cyclin-B/Cdc-2 activity. This cleavage physically and functionally dissociates MEK from ERK activity, thus accounting for the discordant staining we observed.

Applications of PSSAs to Human Neoplasia

Cancer research is the most active field for applications of PSSAs, due to the central role of protein phosphorylation in cancer cell growth and survival signaling. A practical reason for the great progress in this area is that most tumor specimens are rapidly procured by surgical biopsy or resection, rather than at autopsy. A sampling of recent studies in human neoplasms is presented, grouped according to the general class of phosphoprotein target studied (Table 2). Some important phosphoproteins are notably absent, because there are no reports of their immunohistochemical study using PSSAs. An example is c-Kit, which is mutationally activated in mast cell tumors,

chronic myelogenous leukemia, germ cell tumors, and gastrointestinal stromal tumors. Presumably, lack of a suitable specific PSSA has prevented immunohistochemical study of c-Kit activation *in situ*.

Transmembrane Receptor Tyrosine Kinases

ErbB Receptors

One of the first successful applications to human tumors was a monoclonal PSSA to tyrosine-phosphorylated erbB-2 (also known as HER-2/neu). Activation was observed in a subset of breast cancers which overexpress the receptor.²⁸ Similar findings were confirmed in an independent study.²⁹ More recent studies have shown significant correlations between erbB-2 phosphorylation and long-term clinical outcome.^{30,31} Interestingly, erbB-2 was found to be more frequently activated in ductal carcinoma *in situ* than in invasive carcinoma, suggesting that erbB-2 signaling is important in the early stages of breast tumorigenesis.³²

Epidermal Growth Factor Receptor

Although well-documented as an overexpressed or mutated protein in human cancers, relatively few studies of epidermal growth factor receptor (EGFR) phosphorylation in human tumors have been presented. A study of non-small cell lung cancer revealed that phosphorylation, but not overexpression, of EGFR is strongly correlated with worse clinical outcomes.³³ This suggests that assessment of EGFR phosphorylation could be used for therapeutic decision-making, especially for EGFR-directed chemotherapies. Non-seminomatous germ cell testicular tumors also showed significant EGFR activation.³⁴ A recently developed antibody enabled confirmation that the constitutively activated EGFR variant III was also hyperphosphorylated in human lung cancers.³⁵

Insulin-Like Growth Factor 1 Receptor

An antibody specific for the phosphorylated insulin-like growth factor 1 receptor (IGF-1R) was applied to breast carcinoma specimens, suggesting overactivation in cancer cells.³⁶ This antibody was used in a separate study on medulloblastomas, also revealing high levels of receptor phosphorylation relative to normal cerebellum.³⁷ A similar result was obtained in an independent study, which also interrogated the downstream signaling molecules ERK1/2 and AKT, all of which were much more highly phosphorylated in medulloblastoma than control cerebellar tissue.³⁸

Colony Stimulating Factor-1 Receptor

A polyclonal PSSA directed against the macrophage colony stimulating factor receptor (CSF-1R), the product of the c-fms proto-oncogene, was shown to faithfully report activation by ligand in cell culture. Subsequently, application of this antibody to breast tumors revealed

activation in 52% of invasive human breast tumors (72% of CSF-1R-positive cases) in a sample of 114 cases and in 38% of carcinoma *in situ*.³⁹

Intracellular Kinases

ERK/MAP Kinase

The first study to assess activation of a cytoplasmic kinase in archival tumor specimens was performed on human gliomas.¹⁹ PSSAs to ERK/MAPK revealed widespread activation in astrocytomas, regardless of grade, but much lower levels of activation in oligodendrogliomas. Anaplastic progression in oligodendrogliomas was associated with a larger number of cells with active ERK/MAPK. Within glioblastomas, interesting patterns of activation were observed, including around foci of microvascular hyperplasia and necrosis, suggesting possible paracrine signaling relationships. An unexpected observation was that mitotic and actively cycling tumor cells showed diminished activation relative to cells in G₀, suggesting functional roles other than cell proliferation.

ERK/MAPK activation was found to be associated with prostate cancer progression.⁴⁰ Compared to a low level of activation in non-neoplastic prostate, the level of activated ERK/MAPK increased with increasing Gleason score and tumor stage in cancer specimens. Tumor samples from two patients showed no activation of ERK/MAPK before androgen ablation therapy; however, following androgen ablation treatment, high levels of activated ERK/MAPK were detected in the recurrent tumors.

A similar study in breast cancers revealed 72% of tumors to have high nuclear phospho-ERK/MAPK immunostaining relative to normal breast epithelium.⁴¹ Phospho-ERK/MAPK status was found to be a significantly independent predictor for hormonal therapy response duration and patient survival.

Head and neck cancers were found to have elevated ERK/MAPK activation, and expression of the EGFR, its ligand transforming growth factor α , and erbB2 correlated with activation of ERK/MAPK. Activation was also spatially associated with a higher Ki-67 proliferative index.⁴²

Protein Kinase B

Given its widely studied roles in anti-apoptotic signaling, protein kinase B (AKT) has been the subject of several investigations using PSSAs. AKT is normally kept inactive and unphosphorylated by the phosphatase PTEN. In a series of primary epithelial ovarian carcinomas, loss of PTEN expression was highly correlated with elevated AKT phosphorylation.⁴³ A similar inverse relationship was documented in breast cancers and endometrial carcinomas.^{44,45} AKT was found to be frequently activated in non-small cell lung cancers, irrespective of the histological subtypes.⁴⁶ Papillary thyroid cancers also showed evidence of high AKT activation.⁴⁷ A study on bone marrow biopsies from patients with multiple myeloma (MM) demonstrated phospho-AKT staining of malignant plasma cells in a cell membrane-specific pattern,

compared to no staining in non-neoplastic hematopoietic cells⁴⁸.

Transcription Factors and Other Nuclear Proteins

STATs

The STATs (signal transducer and activator of transcription) are a family of key transcription factors involved in cytokine signaling. Constitutive STAT6 phosphorylation was found to be a common and distinctive feature of Hodgkin and Reed-Sternberg cells in classical Hodgkin lymphoma, whereas STAT3 activation was regularly present in both Hodgkin and non-Hodgkin lymphomas.⁴⁹

Multiple myeloma specimens revealed constitutive activation of STAT3 in almost one-half of cases. However, this did not seem to have a major impact on the expression of anti-apoptotic proteins or proliferation.⁵⁰ Prostate carcinomas also show increased phosphorylation of STAT3 relative to non-malignant samples.^{51,52}

β-Catenin

The power of tissue microarray technology was applied to test the expression, localization, and phosphorylation status of β -catenin, a protein involved in both in cell-cell adhesion and the wnt signaling pathway⁵³. Examination of 650 colorectal cancer specimens revealed that the majority of cancers retained some β -catenin membranous staining, whereas cytoplasmic or nuclear expression was seen in 42.5% and 20.4% of specimens, respectively. Phospho- β -catenin showed nuclear staining in only 9.5% of specimens, and there was no apparent membranous or cytoplasmic staining. There was no significant association between β -catenin or phospho- β -catenin and grade or stage. Nuclear expression of phospho- β -catenin, was associated with an improved survival.

Smads

Another tissue microarray study compared the patterns of expression and activation of the Smads in breast cancers.⁵⁴ The Smads are a family of signal transduction molecules that can transmit signals from cell surface receptors for TGF- β to the nucleus. Among 456 cases of human breast carcinoma assembled in tissue microarrays, the majority expressed Smad2, phospho-Smad2, and Smad4. Among patients with stage II breast cancer, lack of phospho-Smad2 expression in the tumor was strongly associated with shorter overall survival.

NF- κ B/I- κ B

In a series of gynecologic squamous lesions, high-grade lesions and carcinomas were found to have increased nuclear translocation of the subunits p50 and RelA as well as loss of I- κ B α immunoreactivity.⁵⁵ Phosphorylation of I- κ B- α occurred in the squamous intraepithelial lesions but not in the advanced stages of squa-

mous cell carcinoma. Since phosphorylation of I- κ B- α is a prerequisite for its degradation, allowing NF- κ B activity, these findings are consistent with activation of the NF- κ B pathway in disease progression.

p53

Given that p53 is regulated by phosphorylation, and the overwhelming quantity of basic and applied research on the role of p53 in human neoplasia, it is surprising how few translational studies have used PSSAs to p53. This may be due in part to the complexity of p53 phosphorylation and the paucity of PSSAs suitable for immunohistochemistry. The majority of transitional cell carcinomas that harbored missense mutations were found to exhibit immunopositivity with a Ser392 phospho-specific p53 antibody.⁵⁶ Phosphorylation at Ser392 activates specific DNA binding functions by stabilizing p53 tetramer formation.

Rb

Given the central and historically important role of this tumor suppressor in cancer research, there are surprisingly few studies on Rb phosphorylation in tumors using PSSAs. A study on uveal melanomas documented increased phosphorylation at carboxy-terminal residues Ser807 and Ser811.⁵⁷ Phosphorylation at these sites was also shown to be important for Rb inactivation in cultured melanoma cells. A study on non-familial retinoblastoma tumors also suggested increased Rb phosphorylation in tumor cells that had not lost expression of the protein, consistent with inactivation.⁵⁸

Histones

Examination of a series of gynecologic squamous intraepithelial lesions using a phospho-histone 1 antibody revealed patterns generally similar to those observed using MIB-1.⁵⁹ However, differing proportion of cells were stained by MIB-1 and the phospho-H1 antibody, suggesting that histone1 phosphorylation, likely due to a cyclin-dependent kinase activity, reveals a different subpopulation of cycling cells.

c-jun

c-jun, a component of the activating protein-1(AP-1) complex, is a target of many growth factor and cytokine signaling pathways. In breast cancer specimens, phosphorylation of c-jun was found to be tightly correlated with ERK1/2 phosphorylation, shortened duration of endocrine response in estrogen receptor-positive patients, but not correlated with proliferation or histological grade.⁶⁰ A study on infiltrating astrocytomas demonstrated elevated phosphorylated c-jun, as well as another AP-1 component, c-Fos in the majority of glioblastomas (grade IV astrocytomas), but not in lower grade (II-III) astrocytomas.⁶¹

Roles for PSSAs in Assessing Efficacy of Signaling Pathway-Targeted Chemotherapies

One of the most exciting potential applications of PSSAs is the assessment of therapeutic efficacy for drugs targeted to specific intracellular signaling pathways. Given the explosion of candidate small molecule inhibitors, and many more in the pharmaceutical pipeline, this is bound to be a growth area. Proof-of-principle for this application was obtained in a xenograft model of human pancreatic adenocarcinoma.⁶² In this study, the efficacy of an epidermal growth factor receptor (EGFR) inhibitor alone and in combination with wortmannin was tested by quantitative immunofluorescence analysis of the phosphorylation state of EGFR, ERK, and PKB. Microscopic quantitation allowed selection of only viable tumor for assay. Immunofluorescence microscopy enabled direct measurement of the ratio of phosphorylated to total kinase protein in the same section, thus normalizing for differences in kinase protein expression. The approach successfully documented the pathway blockade predicted by the drugs administered. The ability to test tumor biopsies for signaling pathway activation state, both before and after initiation of chemotherapy, should allow both prediction of tumoricidal sensitivity and documentation of efficacy in the clinical oncology arena.

A remarkable study used skin biopsies of cancer patients to assess the efficacy of another EGFR inhibitor, ZD1839 (Iressa; AstraZeneca Pharmaceuticals, Wilmington, DE). By using skin as the sentinel tissue in patients with head and neck cancers, the authors were able to show convincingly that the inhibitor blocked not only EGFR phosphorylation, but also reduced downstream ERK/MAPK activation and keratinocyte proliferation index.⁶³ Concomitantly, patients treated with the drug showed increased dermal expression of p27(KIP1) and maturation markers and increased apoptosis. These effects were observed at all dosage levels, before reaching dose-limiting toxicities. The authors suggested that *in vivo* pharmacodynamic assessments using PSSAs on skin biopsies could be used to select optimal effective doses instead of using the maximum-tolerated dose for efficacy and safety trials.

Applications of PSSAs in Degenerative, Inflammatory, and Toxin-Induced Diseases

Phosphorylation state-specific anti-tau protein antibodies have revolutionized the diagnosis and investigation of Alzheimer's disease and a set of distinctive neurodegenerative diseases termed the "tauopathies," including Pick's disease, corticobasal degeneration, progressive supranuclear palsy, progressive subcortical gliosis, and frontotemporal dementia with Parkinsonism. Tau phosphorylation has been the subject of excellent reviews and will not be discussed here.⁶⁴

The ERK/MAPK signaling pathway has been the focus of several translational studies, given its importance in proliferation, differentiation, and survival signaling. A series of non-neoplastic human neurosurgical specimens revealed consistent activation of ERK/MAP kinase in re-

active astrocytes in both subacute and chronic lesions, including infarct, mechanical trauma, chronic epilepsy, and progressive multifocal leukoencephalopathy.⁶⁵ In a mouse model of asbestosis, increased phosphorylated ERK immunoreactivity was found in pulmonary epithelial cells at sites of developing fibrotic lesions after 14 and 30 days of asbestos inhalation.⁶⁶

Insight into the pathogenesis of sporadic inclusion body myositis, a common form of inflammatory myopathy, was obtained by studying MAP kinase pathway activation in muscle biopsies.⁶⁷ Phosphorylated ERK/MAPK, but not JNK or p38 MAPK was detected in the diseased vacuolated fibers that are the hallmark of this disease. The authors hypothesized that the activated ERK/MAPK could be responsible for phosphorylating cytoskeletal proteins that form the filamentous inclusions also characteristic of this disease. A study of human Lewy body diseases, including both classical Parkinson's and diffuse Lewy body disease revealed striking granular cytoplasmic aggregates of phospho-ERK in the substantia nigra, involving almost one-third of surviving neurons, which were largely absent in control cases. Double-labeling studies and examination of preclinical cases suggested that these phosphorylation changes might occur relatively early in the disease process, preceding actual Lewy body formation.⁶⁸

Non-neoplastic inflammatory diseases are largely unstudied using PSSAs. Inflammatory bowel disease tissues revealed activation of the STAT1 pathway in inflammatory cells of ulcerative colitis, but not in Crohn's disease or normal colon, suggesting disease-specific signaling mechanisms.⁶⁹

An unexpected connection between the trinucleotide repeat diseases and aberrant protein phosphorylation was suggested by a study using a general phosphoserine antibody.⁷⁰ Anti-phosphoserine staining of tissue sections from brains affected by trinucleotide repeat disease revealed high reactivity in neuronal inclusions and affected nuclei, but not normal neurons. The regional distribution of the phosphorylated nuclei in neurons correlated with other pathological changes. Using the same antibody to immunopurify the phosphorylated protein(s) in neuronal inclusions, the authors found the major phosphoprotein to be histone H3. This finding has interesting implications for potential mechanisms by which the trinucleotide repeat gene products could interfere with gene transcription.

Another disease characterized by abnormal protein aggregation is alcoholic hepatitis, in which cytokeratins 8 and 18 accumulate as cytoplasmic inclusions (Mallory bodies). To investigate roles for cytokeratin phosphorylation in Mallory body formation, PSSAs to cytokeratins were used to probe diseased and normal liver tissue sections.⁷¹ Hepatocyte cytokeratins were found to be hyperphosphorylated at multiple sites in a Mallory body mouse model, even in hepatocytes that had yet to develop Mallory bodies. Again the suggestion was that aberrant hyperphosphorylation might precede inclusion formation.

Concluding Remarks

Studies of protein phosphorylation previously ignored the spatial complexity inherent in tissue and cell signaling. The advent of PSSAs now makes possible the probing of intact tissues and cells, both in experimental models and human specimens, for patterns of specific protein phosphorylation. In studies on tumor biology, PSSAs will allow analysis of protein phosphorylation within neoplastic cells independently of the non-neoplastic stroma. The potential complexity of staining patterns obtained when using PSSAs raises interpretive problems. Comparison of results of studies using PSSAs will require some level of quantitation, such as is routine with proliferation markers such as Ki-67. This will be straightforward where staining is discrete and easily separated from background, such as a nuclear transcription factor, but may be less objective when the staining pattern is more complex. For example, a PSSA may label both angiogenic blood vessels as well as tumor cells, so it would not be appropriate to report results simply as "percentage of cells positive."

An additional layer of complexity may appear as we learn more about the functional significance of multiple phosphorylation sites on single proteins. Evidence for differential phosphorylation of adjacent tyrosine residues of the erbB2 receptor in breast cancers raises the issue of heterogeneity at the molecular as well as cellular level.^{72,73}

PSSAs may prove to be especially useful for demonstrating chemotherapeutic efficacy, when the targets are known players in phosphorylation cascades. Applications of PSSAs to non-neoplastic diseases are in their infancy, but show great potential. Just as the development of comprehensive gene chips has allowed near genome-saturating studies of gene expression, the eventual development of PSSAs for virtually all phosphoproteins will make feasible a similar large-scale approach to phosphorylation cascades. Novel applications of PSSAs, especially in combination with the power of tissue microarrays,⁷⁴ should reveal new paradigms for disease classification and diagnosis based on protein activation state.

Acknowledgments

I thank Maja Zecevic for collaboration on the unpublished mitotic MEK phosphorylation studies, and all of the members of Michael Weber's laboratory for guidance and insightful discussions on PSSAs and cell signaling in general. I am grateful to Scott VandenBerg for ongoing discussion and support. Dan Heffron provided expert technical assistance.

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