Site-Specific ^{32}P -Labeling of Cytokines, Monoclonal Antibodies, and Other Protein Substrates for Quantitative Assays and Therapeutic Application

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ABSTRACT

Radiolabeled proteins are used in a variety of laboratory applications as well as in radioimmunotherapy. This review focuses on methods that utilize genetic engineering to introduce exogenous phosphorylation sites into proteins. Protein kinase substrate sites can be introduced into target proteins to serve as tags for several purposes. Because many protein kinases, each preferring a unique consensus sequence, are well characterized, the essential structure and function of the target protein can be effectively preserved through judicious selection and design of the phosphate incorporation site. After phosphorylation, these proteins are often indistinguishable from the parent molecules in assays of functional or biological activity. This convenient approach permits incorporation of ^{32}P , ^{33}P , ^{35}S , or nonradioactive ^{31}P , and is rapid, efficient, and safe. Most importantly, ^{32}P labeling of monoclonal antibodies or other therapeutic protein candidates has several significant advantages over radioiodination or chemical conjugation of heavy metal isotopes.

INTRODUCTION

Radiolabeled proteins have proven to be increasingly valuable in a wealth of applications. These range from basic biology and biochemistry (e.g., delineation of protein-protein interactions, protein-DNA, and protein-RNA interactions), to application to human disease (e.g., pharmacokinetics, diagnostic imaging, and radiotherapy). In the field of protein-protein interactions alone, radiolabeled proteins are essential tools in assessing antigen-antibody affinities, receptor-ligand interactions, receptor internalization and degradation, and expression cloning of receptors. In the laboratory setting, ^{125}I is often the tag of choice for rapidly labeling proteins to high radiospecific activity. However, the risks and drawbacks of radioiodination are well known. Because iodinated proteins undergo dehalogenation, thyroid gland monitoring of lab personnel is required. The high-energy γ emission of ^{125}I necessitates increased protective measures including lead shielding. Furthermore, iodination is a random process occurring throughout the protein chain, resulting in a heterogeneous population of labeled proteins. In fact, iodination typically results in significant loss of activity of the target protein, an effect that has been particularly well documented in the case of monoclonal antibodies.

To circumvent the multiple drawbacks of iodination, we sought to develop a gentler, site-specific, high radiospecific activity labeling procedure that would allow the bulk of protein bioactivity to be retained. Secondly, we set out to use an isotope with a better safety profile than that of radioactive iodine. We decided upon enzymatic phosphorylation through engineered kinase sites as this approach avoids, in theory, many of the complications inherent in iodination and in chemical conjugation of heavy metal isotopes. Both the vector construction and subsequent labeling procedures using analogs of ATP are convenient and rapid and can be applied to a variety of protein targets.

Our initial work in this area focused on modification of interferons for use in receptor binding experiments (47,82,88). When human interferon αA was used as a model and re-engineered to contain kinase substrate domains, neither immunoreactivity nor biological activity was adversely affected, either in the presence or absence of the phosphate label. These results suggested that this approach could yield novel radioligands suitable for radioimmunoassays, ligand-binding studies, and possibly pharmacokinetic evaluation. Subsequently, we modified several other cytokines and monoclonal antibodies, and have now begun to evaluate the therapeutic potential of some of these molecules.

The engineering and labeling procedures were validated for cAMP-dependent protein kinase (PKA) (47–49,82,88), casein kinase I (52), casein kinase II (51), and Src tyrosine kinase (Src) (50) sites. Two overall strategies became immediately evident: attachment or insertion of a kinase domain tag to the N or C terminus or internal to the target protein, or minor modification of endogenous sequences in the protein to yield a kinase substrate site. The latter approach is further enabled by the availability of different kinase sites allowing the insertion of acidic (casein kinase I and II), basic (PKA), or neutral (Src) residues as needed in order to minimally modify the target protein. That is, the engineering of the phosphorylation site can be optimized to have the minimum impact on the pI and hydrophobicity of the

protein such that tertiary and quaternary structure and general pharmacokinetic properties are preserved.

For applications such as radioimmunotherapy, a number of isotopes commonly have been coupled to tumor-specific monoclonal antibodies, thus increasing their lethality to tumor cells. In addition to iodination with ^{131}I , chelation of heavy metal isotopes such as ^{90}Y , ^{67}Cu , and ^{188}Re have each been examined for therapeutic monoclonal labeling. However, immunogenicity of therapeutic monoclonal antibodies often limits their therapeutic potential (21,76), a concern that arises with any chemical conjugation procedure. Whereas the insertion of kinase substrate site into a monoclonal can maintain the essential quaternary structure of the molecule, chemical conjugations of iodine, peptides, or chelators are inherently damaging processes. Neither iodination chemistry nor chemical coupling of chelating agents or peptides is site specific. These reactions then yield multiple new epitopes on the resultant antibody bearing any of these radiolabels. Therefore, the therapeutic potential of these agents is compromised, particularly with regard to re-administration upon which the immune system could rapidly eliminate the protein from the circulation. The strategy of chemically conjugating PKA-phosphorylatable peptides to a larger protein (18) is remarkably similar to the standard coupling approach used to make peptide-specific antisera. Clearly, the peptide presents a new epitope, as does the linkage site to the larger protein. The immunogenicity of the larger protein is also exacerbated by peptide linkage (11).

Non-site-specific conjugation chemistries such as iodination also adversely affect specificity of the monoclonals for the tumor-specific antigens, possibly through derivitization of amino acid side chains at or proximal to the complementarity/specificity determining regions necessary for antigen recognition. In addition, the risk of free iodine concentrating in patient or healthcare worker thyroids, stomachs, and salivary glands also instigated our pursuit of the phosphorylation approach. The use of engineered kinase sites, while conferring some potential for immunogenicity, appears to have significant advantages in preparing radiolabeled proteins for therapeutic application. This approach leaves largely intact the general structure of the target protein and permits selection of a specific kinase to minimize the impact of sequence mutations on protein function.

PROTEIN KINASE REACTIONS

The transfer of phosphate from the γ phosphate of ATP or other nucleoside triphosphates to an acceptor amino acid can be accomplished by a variety of phosphotransferase enzymes. Protein-serine/threonine and protein-tyrosine kinases have been particularly well characterized, although other amino acid side chains can also be phosphorylated by specific protein kinases (26,27). Many of these transferases are highly efficient *in vitro*, displaying attractive K_m , k_{cat} , and turnover numbers. Although ectokinases are less well characterized, they may prove to be attractive reagents for this purpose as these enzymes have been found to be released from neutrophils (77) and on the surface of T-lymphocytes (73), suggesting that the substrate sites for these kinases may possess low immunogenicity.

Because of their wide availability and extensive literature

base, protein-serine/threonine and protein-tyrosine kinases and their substrates will be the focus of this review. These kinases transfer the γ phosphate or sulfate from ATP or ATP γ S or their analogs, and thus can be used to label proteins with radioactive ^{32}P , ^{33}P , ^{35}S , or nonradioactive ^{31}P . The longer half-life of ^{35}S (87 days) may provide advantages in the laboratory setting, although the low energy beta emission is insufficient for therapy. The physical half-life (14.2 days) and energetic beta emission (mean 695 keV) of ^{32}P are appealing properties in terms of therapeutic use with monoclonal and other protein substrates. If higher radiospecific activity is warranted, multiple phosphorylation sites or cassettes can be incorporated into the expression vector. In contrast, multiple iodination of single protein molecules would significantly increase the risk of protein inactivation in the conjugation reaction. For those applications such as pharmacokinetics where radioactivity may not be warranted, ^{31}P -labeled proteins can be followed by phosphotyrosine-, phosphothreonine-, and phosphoserine-specific antibodies or by straightforward mass spectroscopy.

As mentioned above, two methods are immediately apparent for introducing kinase substrate sites into target proteins: fusion and site-specific mutation. In either case, access of the kinase to the recognition motif is a consideration such that surface epitopes or tail epitopes are typically the preferred sites on which to design the addition of a phosphate. One may also consider the potential impact of an introduced phosphate if inserted at or proximal to known regions of protein-protein interaction. In fact, the demonstration of an effect of phosphorylation within such a region on a well-characterized protein contact interface may yield new information on the dynamics of that interaction. Depending on the intended use of the modified protein, phosphorylation consensus sequences or cassettes can be fused to the N or C terminus or inserted internally within the protein. Alternatively, near-consensus site homologies within the target protein can be exploited through the site-specific mutation approach. That is, it is often possible to construct a new phosphorylation site within the protein through mutation of just one or a few amino acids. These manipulations are easily performed through standard genetic engineering techniques.

We used the technique of site-specific mutation to introduce phosphorylation sites into the amino acid sequences of a number of interferons (47,48,82) (also R. Donnelly and S. Pestka, unpublished data for IL-2; W. Wu and S. Pestka, unpublished data for monoclonal antibody chCC49-WW5). The fusion approach was also useful for a variety of proteins and kinase sites (1,3–7, 9, 15,19,28,32,33,36,49–52,59,60,62,63,68,74,81,83,85,86,88): interferon (88), monoclonal antibodies (49–52), retinoblastoma protein (1,32), osteopontin (4), c-Fos (5), calmodulin (15), diphtheria toxin (59), endothelial growth factors (59), enterotoxins, (59) lymphokines (59), ricin (19,59), antibody fragments (62,63), microtubule associated protein (81), *Escherichia coli* RNA polymerase β' and σ subunits (3,6), green fluorescent protein (85), *E. coli* TraT-RHO surface protein fusion (7), bacteriophage T4 primase (28), erythropoietin (68), opioid receptor peptide ligands (36), interleukin receptor subunit extracellular domains (83), IL-3 (C. Miyamoto, personal communication), interferon β (X.-X. Zhou, J. Langer, and S. Pestka, unpublished data), IL-10, vIL-10, and cmvIL-10 (L. Izotova, S. Kotenko, S. Saccani, and S. Pestka, unpublished observations). Where exam-

ined, the activities of the modified and phosphorylated proteins were, in most cases, virtually indistinguishable.

Another approach developed by Foxwell et al. (18) introduced a kinase recognition site into a peptide that was then covalently linked to a monoclonal antibody. Whereas the phosphorylated monoclonal was stable in serum, proteins modified by this method would have limited potential in vivo as this approach appears to favor the generation of a heterogeneous product carrying multiple new epitopes, thus enhancing antigenicity.

PROTEIN KINASE SUBSTRATE SITES

Consensus phosphorylation sequences have been identified and reported for a wide variety of kinases. We have successfully used four kinases to date by way of the recognition sequences shown in Table 1 that were based on the expansive kinase literature (for examples, see References 13,20,25,34,39,53,56–58, 64,87). Because protein serine/threonine kinases show significant differences in K_m for serine vs. threonine (35), we utilized only serine as the phosphate acceptor to take advantage of the higher efficiency of this phosphotransferase reaction.

PROTEINS WITH INTRODUCED PHOSPHORYLATION CONSENSUS SEQUENCES

Our initial reports demonstrated that gentle phosphate labeling of interferons was not detrimental to protein structure or function (47,82,88). Binding and crosslinking studies were carried out using phosphate-labeled human interferon (IFN) α A, α B2, and α A/D (2,10,16,47,66,78,82,88). A number of groups have followed similar procedures to introduce phosphorylation sites into a variety of proteins (Table 2). Blonar et al. (5) designed an expression vector to encode proteins with amino terminal extensions containing a cAMP-dependent protein kinase (PKA) recognition site. This region was fused to the leucine zipper dimerization motif of c-Fos that was then used to screen a cDNA library. The protein-protein interactions of the phosphorylated protein were then examined. Kaelin et al. (32) used the vector prepared by Blonar et al. (5) to prepare a 32 P-labeled glutathione S-transferase (GST)-retinoblastoma (pRB) fusion protein for efficient screening of expression libraries. Continuing these studies, Adams et al. (1) determined the region of pRB phosphorylated by cyclin A-cdk2 and cyclin E-cdk2. Ron and Dressler (74) constructed an expression vector similar to that of Blonar et al. (5) and Kaelin et al. (32). The vector encoded a GST tag and a PKA site that could be fused to target proteins. The 32 P-labeled fusion protein could be used to identify interacting proteins. Stofko-Hahn et al. (81) constructed a vector with a PKA site, a Factor Xa cleavage site, and a calmodulin-binding peptide. Fusion proteins could easily be purified on a calmodulin affinity matrix and the fused protein phosphorylated with [γ - 32 P]ATP and PKA. Chen and Hai (9) designed an *E. coli* expression vector with two tandem repeats of the heart muscle kinase consensus sequence and a His₆ tag designed to express fusion proteins for protein-protein interaction studies. Similarly, Kelman et al. (33) constructed *E. coli* expression vectors encoding a PKA substrate domain fused to hemagglutinin influenza

Table 1. Recognition Sites for Various Protein Kinases

Protein Kinase	Recognition Site
cAMP-dependent protein kinase (PKA)	ArgArgXaaSerXab
Casein kinase I	AspAspAspAspSer IleAsp AspAsp- AspAspSer
Casein kinase II	AspAspAspAspSerGluGluAsp
Src tyrosine kinase	LysValGluLysIleGluGluGlyThrTyr- GlyValValTyrLys
<p><i>Note:</i> For introduction of the cAMP-dependent protein kinase recognition site, we used ArgArgXaaSerXab where Xaa is usually Ala and Xab has been Val, Leu, Met, or Gln (47–49,82). However, as shown previously, a wide variety of amino acids can substitute for Xaa and Xab effectively (2,35,66,78,84). For the casein kinase I site, we used two AspAspAspAspSer sequences in tandem, connected with IleAsp shown in boldface (52) based on sequences reported by Meggio et al. (58). For the casein kinase II site, we used (51) the sequence shown (2,34,61,65,67). For the Src tyrosine kinase site, we used the sequence shown (50) based on the sequence reported (8,50).</p>	

virus epitope (HA) and His₆ tags. This enabled fusion proteins to be concentrated by immunoprecipitation, purified on metal affinity chromatography, and labeled with isotope using the respective tags. Useful substrates for a number of kinases were constructed by Yang et al. (85) as fusion construct with green fluorescent protein (GFP). This work yielded a particularly efficient substrate for Src tyrosine kinase. To study the binding and engulfment of bacteria by BHK21 cells, Chang and Lo (7) fused a PKA substrate site onto the *E. coli* outer membrane lipoprotein TraT fused with snake venom rhodostomin (RHO) and a thrombin cleavage site. The fusion protein was expressed on the bacterial surface and could be radiolabeled by PKA. Surface-radiolabeled bacteria then permitted the development of a rapid, high sensitivity quantitative assay of bacterial binding to and internalization by BHK21 cells. Jing et al. (28) fused heart muscle kinase to the N or C terminus of the gp61 primase subunit of the bacteriophage T4-coded primosome. The fusions were then evaluated for phosphorylation in the presence or absence of single stranded DNA. Single-stranded DNA (ssDNA) protected both the N- and C-terminal fusions from phosphorylation, suggesting that a specific interaction of these regions with ssDNA shielded the phosphorylation sites from the protein kinase. Kinase protection was further enhanced as more components of the primosome were included in the assay.

Ashkar et al. (4) prepared a fusion protein with osteopontin containing a kinase recognition site and GST to study function and posttranslational modifications of mammalian osteopontin. Moss et al. (60) prepared fusion proteins with the intracellular domains of the γ -aminobutyric acid type A (GABAA) receptor to determine the recognition sites for PKA and protein kinase C

REVIEW

Table 2. Proteins Labeled by Incorporation of Phosphorylation Sites

Protein	Protein Kinase Site	References
Human IFN- α A	PKA	47,88
Human IFN- α B2	PKA	82
Human IFN- α A/D	PKA	82
Human IFN- β	PKA	X.-X. Zhao, J. Langer, and S. Pestka, unpublished data
Bovine IFN- τ	PKA	2
Human IL-2	PKA	R. Donnelly and S. Pestka, unpublished
Human IL-3	PKA	C. Miyamoto, personal communication
Human IL-10	PKA	L. Izotova, S. Kotenko, S. Saccani, and S. Pestka, unpublished data
vIL-10, cmvIL-10	PKA	L. Izotova, S. Kotenko, S. Saccani, and S. Pestka, unpublished data
c-Fos	PKA	5
Glutathione S-transferase	PKA	4,15,32,74
Retinoblastoma protein	PKA	32
Calmodulin	PKA	15
Calmodulin binding peptide	PKA	81
Osteopontin	PKA	4
γ -Aminobutyric acid receptor A	PKA	60
Dopamine receptors	PKA	86
<i>E. coli</i> RNA polymerase β' subunit	PKA	3,6
<i>E. coli</i> RNA polymerase σ subunit	PKA	3,6
SV40 large T-antigen	PKA	84
	Casein kinase II	
	Cyclin-dependent kinase	
Antibody fragments	Casein kinase II	62
Ricin A chain	PKA	19,59
Diphtheria toxin amino terminal fragments	PKA	59
Ricin A/diphtheria toxin hybrid	PKA	59
Toxic shock syndrome toxin I	PKA	59
Vascular endothelial growth factor isoforms	PKA	59
Anti-TAG-72 monoclonal antibody	PKA	48,49
Anti-TAG-72 monoclonal antibody	Casein kinase I	52
Anti-TAG-72 monoclonal antibody	Casein kinase II	51,61,62
Anti-TAG-72 monoclonal antibody	Src tyrosine kinase	50
Green fluorescent protein	PKA	85
	Src tyrosine kinase	
	c-Abl	
	Protein kinase A	
	Protein kinase C β II	
<i>E. coli</i> /TraT-RHO hybrid	PKA	7
Bacteriophage T4-coded primase (gp61)	Heart muscle kinase	28
Anti-CEA single chain monoclonal antibody	PKA	63
Erythropoietin		68
Enkephalin and deltorphin	PKA	36
IL-1 receptor and IL-2 receptor α subunit extracellular domains	PKA	83

Note: Phosphorylation sites were introduced into various proteins as shown. PKA, cAMP-dependent protein kinase; IFN, interferon; IL, interleukin; vIL-10, the viral homologue of human IL-10; CEA, carcinoembryonic antigen.

(PKC). Similarly, Zamanillo et al. (86) used fusion proteins of the dopamine receptor subtypes to study their phosphorylation by PKA and PKC. Xiao et al. (84) constructed fusion proteins with recognition sites for PKA, casein kinase II, and the cyclin-dependent kinase cdc2. This group demonstrated that nuclear import of SV40 large tumor antigen fusion proteins, normally regulated by the motif comprising phosphorylation sites for casein kinase II and the cyclin-dependent kinase cdc2, could be controlled by PKA if the PKA recognition site is substituted for the casein kinase II site. Fischer et al. (15) constructed a vector that contained sequences encoding a FLAG epitope, GST, and a PKA recognition site, and fused these to the coding sequence of calmodulin. The ^{32}P -labeled calmodulin fusion protein was used to identify calmodulin-binding proteins in cellular extracts to replace laborious techniques employing ^{125}I -calmodulin or biotinylated calmodulin. Arthur and Burgess (3) and Burgess et al. (6) used N-terminal His₆-PKA fusions of *E. coli* RNA polymerase β' and σ subunits to map the interaction site between one of the σ subunits and the β' subunit of the core enzyme.

Neri et al. (61,62) developed protein kinase tags for antibody fragments secreted from bacteria to identify immunoreactivity. After the fragments were labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and casein kinase II, the antibody fragments were used to determine affinity for specific antigens. These investigators concluded that "in contrast to non site-specific methods such as radioiodination, antibodies labeled with casein kinase II retain full immunoreactivity" (62). Patrick et al. (63) developed a ^{32}P -labeled single chain monoclonal antibody against carcinoembryonic antigen (CEA). The labeled fusion construct showed selective toxicity and rapid internalization in LS-174T cells expressing high levels of CEA.

Mohanraj et al. (59) prepared an expression vector for introduction of a PKA site at the amino terminus of proteins. Seven different phosphorylatable proteins were prepared, demonstrating the generality of the methodology. After labeling with ^{32}P , they concluded that "all seven proteins used in this study could be expressed with the phosphorylation sequence at their amino terminus and specifically labeled without loss of biological activity" (59). Erythropoietin bearing an N-terminal protein kinase domain was shown by Quelle et al. (68) to retain full receptor binding activity and biological activity in vitro, and could be used for receptor internalization studies. In contrast, C-terminal kinase substrate domain fusions failed to compete for receptor binding. Kieffer et al. (36) used a PKA tag fused to the C terminus of the delta opioid-selective peptides enkephalin and deltorphin to quantify affinity of these ligands for the receptor. Whitehorn et al. (83) fused the extracellular domains of the IL-1 receptor, IL-2 receptor, and E-selectin to a 3' PKA consensus sequence. After labeling with ^{32}P by PKA treatment, the fusion proteins competently bound their cognate ligands.

PURIFICATION OF MODIFIED PROTEINS

Because the introduction of a carefully designed phosphorylation site usually does not materially alter the structure or physical characteristics of the target protein, the same procedures used for purifying the unmodified protein have been found to be effective prior to phosphorylation (47–52,82,88).

PHOSPHORYLATION OF PROTEINS BY PKA

To attain high levels of labeling, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ should be used at the highest specific activity available. This radiolabel is supplied in aqueous (0.01 M Tricine) buffer. The entire contents of the vial are dried in a SpeedVac® (Thermo Savant, Holbrook, NY, USA) and the residue dissolved in a minimal volume of water to the final concentration denoted below. The catalytic subunit of the cAMP-dependent protein kinase from bovine heart is used for the reaction illustrated below using a monoclonal antibody MAb-ch-CC49-6P and MAb-ch-CC49-KI.

MAb-ch-CC49-6P is labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and PKA as described previously (49). Approximately 10 μg of the monoclonal were incubated at 30°C for 60 min with 0.5 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity of 6000 Ci/mmol; DuPont-NEN, Boston, MA, USA) and 25 units of the catalytic subunit of bovine heart muscle PKA (specific activity of $> \text{or} = 20\,000$ units/mg [Sigma, St. Louis, MO, USA]; dissolved in 6 mg/mL DTT) in 25 μL of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and 12 mM MgCl_2 , then cooled on ice to stop the reaction. After addition of 300 μL of 5 mg/mL bovine serum albumin in 10 mM sodium pyrophosphate, pH 6.7 at 4°C, the 0.325 mL reaction mixture was dialyzed against 1000 volume of phosphate-buffered saline (PBS) overnight at 4°C. Dialysis buffer was changed twice. Incorporation of radioactivity into the monoclonal antibody was measured with a liquid scintillation spectrometer after precipitation of the protein with trichloroacetic acid (TCA) (65). To remove labile ^{32}P , the final product in 0.325 mL was adjusted to pH 7.4 with 1 M Tris base, then incubated at 37°C overnight. The labeled material was then stored in a liquid nitrogen freezer in small aliquots until use.

After the reaction, the samples were dialyzed as noted previously. However, alternative methods for removal of free phosphate and ATP can be used, such as centrifugal filter separators or disposable gel filtration columns. Although all of these procedures are effective, we found that dialysis is the simplest and most effective method. It should be noted that we also observed two rates of phosphate release (48–52). One was the rapid release reflecting loosely bound phosphate with the major attached phosphate being relatively stable. When this is observed, we carry out the overnight dialysis at 37°C rather than at 4°C. As expected, autoradiography shows that the final phosphorylated product comigrates with the corresponding Coomassie blue-stained protein band upon polyacrylamide electrophoresis. When fusion proteins containing epitope tags such as FLAG, Myc, HA, or GST are used, the protein can be isolated on beads bearing an antibody or ligand with high affinity for these tags. After washing the beads, phosphorylation of the protein can be performed in situ on the bead-immobilized protein. After washing to eliminate free kinase, nucleotide, ^{32}P , and other contents of the reaction mix, phosphorylated proteins can be released from the affinity column by elution with free affinity ligand or by acidification.

It is common for this procedure to yield protein products having very high radiospecific activity, often at the level of 5000 Ci/mmol. The specific activity is further raised by inserting more than one phosphorylation site cassette into the target protein (49). Additional specific reagents and further details on these procedures are described elsewhere (67).

PHOSPHORYLATION OF PROTEINS BY CASEIN KINASE I

MAB-chCC49CKI was labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and casein kinase I as described (52). Approximately 10 μg of the monoclonal were incubated at 37°C for 60 min with 0.5 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity of 6000 Ci/mmol) and 15 units of casein kinase I (Upstate Biotechnology, Waltham, MA, USA; Cat. No. 14-112) in 25 μL of 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 12 mM MgCl_2 , then cooled on ice to stop the reaction. After addition of 300 μL of 5 mg/mL bovine serum albumin in 10 mM sodium pyrophosphate, pH 6.7 at 4°C, the 0.325 mL reaction mixture was dialyzed against 1000 volumes of PBS overnight at 4°C. Dialysis buffer was changed twice. Incorporation of radioactivity into the monoclonal antibody was measured with a liquid scintillation spectrometer after precipitation of the protein with TCA (65). To remove labile ^{32}P , the final product was preincubated at 37°C for 24 h in the medium in which it was to be used or in pH 7.4 buffer such as PBS. As noted above, this procedure was established because it was apparent that a percentage of the ^{32}P phosphate attached to the protein was labile and could be eliminated by this preincubation (48–52).

PHOSPHORYLATION OF PROTEINS BY CASEIN KINASE II

MAB-chCC49CKII was labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and casein kinase II as described (51). Approximately 10 μg of the monoclonal were incubated at 30°C for 60 min with 0.5 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity of 6000 Ci/mmol) and 1 μL (0.23 units) of casein kinase II (Upstate Biotechnology, Cat. No. 14-197) in 25 μL of 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, and

12 mM MgCl_2 , then cooled on ice to stop the reaction. After addition of 300 μL of 5 mg/mL bovine serum albumin in 10 mM sodium pyrophosphate, pH 6.7 at 4°C, the 0.325 mL reaction mixture was dialyzed against 1000 volumes of PBS overnight at 4°C. Dialysis buffer was changed twice. Incorporation of radioactivity into the monoclonal antibody was measured with a liquid scintillation spectrometer after precipitation of the protein with TCA (65). To remove labile ^{32}P , the final product was preincubated at 37°C for 24 h in the medium in which it was to be used or in pH 7.4 buffer such as PBS. As noted above, this procedure was established because it was apparent that a percentage of the ^{32}P phosphate attached to the protein was labile and could be eliminated by this preincubation (48–52).

PHOSPHORYLATION OF PROTEINS BY Src TYROSINE KINASE

MAB-chCC49Tyr was labeled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and casein kinase II as described (50). Approximately 10 μg of the monoclonal were incubated at 30°C for 60 min with 0.5 mCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (specific activity of 6000 Ci/mmol) and 15 units of Src tyrosine kinase (Src kinase, P60^{c-src}; Upstate Biotechnology, Cat. No. 14-117) in 25 μL of 50 mM Tris-HCl, pH 7.0, and 50 mM MgCl_2 , then cooled on ice to stop the reaction. After addition of 300 μL of 5 mg/mL bovine serum albumin in 10 mM sodium pyrophosphate, pH 6.7 at 4°C, the 0.325 mL reaction mixture was dialyzed against 1000 volumes of PBS overnight at 4°C. Dialysis buffer was changed twice. Incorporation of radioactivity into the monoclonal antibody was measured with a liquid scintillation spectrometer after precipitation of the protein with TCA (65). To remove labile ^{32}P , the final product was preincubated at 37°C for 24 h in the medium in which it was to be used or in pH 7.4 buffer such as PBS. As noted above, this procedure was established because it was apparent that a percentage of the ^{32}P phosphate attached to the protein was labile and could be eliminated by this preincubation (48–52).

BINDING OF ^{32}P -LABELED MONOCLONAL ANTIBODIES TO CELL SURFACE ANTIGENS

Binding of ^{32}P MAB-chCC49KI and ^{32}P MAB-chCC49-6P to MCF-7 4C10 human breast carcinoma cells was performed as described (49). MCF-7 4C10 cells used for binding studies were grown to confluence in 6-well tissue culture plates in medium (DMEM; GIBCO/Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 0.05 mg/mL sodium pyruvate, 0.005 mg/mL insulin, 0.5 $\times$ nonessential amino acids, and treated with 1000 units/mL human interferon γ for 48 h prior to assay. Twenty minutes before assay, the medium was removed, and 1 mL of fresh medium was added to each well. Then ^{32}P MAB-chCC49KI was added with or without a 100-fold excess of unlabeled MAB-chCC49KI or MAB-chCC49. Following incubation at room temperature for 1 h, the plates were washed four times with 2 mL of ice-cold PBS to remove the unbound radioactive ligand. One milliliter of 1% sodium dodecyl sulfate (SDS) was added to each well to remove cells and the bound ^{32}P -labeled monoclonal. Radioactivity

Figure 1. Binding of monoclonal antibody chCC49K1 to MCF-7 4C10 breast carcinoma cells. Labeling of chCC49K1 monoclonal antibody and binding to MCF-7 4C10 cells were performed as described in the text (49). Nonspecific binding values were subtracted from total bound counts to obtain specific bound counts shown on the ordinate. Reproduced, by permission of the publisher, from Lin et al. (49).

bound by the cells was determined in a liquid scintillation counter by placing the 1 mL sample in 5 mL of scintillation fluor. Binding of [32 P]MAB-chCC49-6P to MCF-7 4C10 cells was determined similarly with the exception that the cells were cultured in 96-well plates since the radiospecific activity of this monoclonal was greater than that of [32 P]MAB-chCC49KI.

The binding of the monoclonal antibodies to TAG-72 antigen on the MCF-7 4C10 breast cancer cell line is shown in Figure 1. [32 P]MAB-chCC49KI bound to MCF-7 4C10 cells treated with 1000 units/mL human interferon γ with specific binding approaching saturation at the higher concentrations of antibody tested. Scatchard analysis (75) of the data indicated that these cells have 1900 receptors per cell as calculated by the binding of [32 P]MAB-chCC49KI. The binding of [32 P]MAB-chCC49KI to the cells was blocked by the addition of unlabeled MAB-chCC49KI. The binding of [32 P]MAB-chCC49-6P to this cell line (data not shown) was similar to that of [32 P]MAB-chCC49KI. It is noteworthy that specific binding was very nearly identical to total binding when using these 32 P-labeled monoclonals. This result was initially surprising when compared to results with 125 I-labeled proteins. It is fortuitous that once this is demonstrated for a particular binding assay/monoclonal pair, 32 P-labeled proteins can often be studied without competitor, thus saving significant amounts of expensive and difficult to obtain unlabeled protein. However, in developing a binding assay,

control experiments need to be performed to determine whether nonspecific binding is a significant contributor to total binding.

BINDING OF 32 P-Labeled INTERFERON TO CELL STRUCTURE RECEPTORS

The binding of 32 P-labeled human IFN α B2 and IFN α A/D to bovine MDBK cells and human Daudi cells is shown in Figure 2. Again, it can be noted that specific binding is nearly identical to total binding for each of these interferons on each cell line. We hypothesize that the extremely low nonspecific binding is largely conferred by the gentle enzymatic labeling of the monoclonals, as well as the labeling of the antibodies at highly specific sites. Depending on the labeling procedure, chemical labeling of proteins occurs on random lysine or tyrosine residues. This random labeling can inactivate a portion of the protein molecules, often resulting in denaturation of the protein and thus contributing to the higher nonspecific labeling seen in radioiodinated proteins. Another contributor to the higher nonspecific binding observed with iodination is that the iodine atom is large and nonpolar, and effectively enhances nonspecific hydrophobic interactions. Furthermore, chemical labeling results in a mixed population of derivitized molecules having the label attached at a variety of locations along the protein chain.

Figure 2. Binding of 32 P-labeled human IFN- α B2 and human IFN- α A/D to cells. 32 P-Hu-IFN- α B2 or 32 P-Hu-IFN- α A/D was added to cells at the indicated concentrations in the absence (total binding, open circles) or presence (nonspecific binding, open triangles) of excess nonradioactive Hu-IFN- α B2 or Hu-IFN- α A/D, respectively, and incubated for 1 h at 24°C. Specific binding (filled circles) represents the difference between the total and the nonspecific binding. (A and B) Binding of 32 P-Hu-IFN- α B2 to human Daudi and Bovine MDBK cells, respectively; (C and D) binding of 32 P-Hu-IFN- α A/D to Daudi and MDBK cells, respectively. (Inset) Scatchard analysis of the specific binding data (B, radioactivity of ligand bound to cells; F, radioactivity of free or unbound ligand). Reproduced, by permission of the publisher, from Wang et al. (82).

Site-specific labeling of proteins via kinase site insertion circumvents these drawbacks of random chemical conjugation. Furthermore, the availability of different protein kinases permits conservation of positive, negative, or neutral charge and contributes to retaining the activity of the protein.

STABILITY OF PHOSPHORYLATED PROTEINS

As noted above, some phosphorylated proteins exhibit two rates of radiophosphate loss, a rapid rate and a relatively slow rate of loss of “stable phase” phosphate (Figure 3). When this rapid rate is seen, incubating the phosphorylated product at 37°C during the dialysis procedure for about 24 h eliminates the rapid decay phase (Figure 4). The percentage of [32 P]phosphate retained on the MAb at different intervals was compared to the initial value in either buffer or various sera (Figure 4). Approximately 93%–96% of the radiophosphate remained attached to the MAb after 24 h incubation in buffer, fetal bovine serum, human serum, or mouse serum.

MOLECULAR MODELING TO DESIGN APPROPRIATE SITES OF PHOSPHATE INTRODUCTION

Because the crystal structures of many proteins are known, it is possible to use the coordinates of the crystal structure to eval-

uate the effects of the insertion of the phosphorylation site and the phosphate itself. We have been able to utilize molecular modeling very effectively to predict useful sites within a protein in which to introduce phosphorylation sites with minimal modification of the protein structure (W. Wu and S. Pestka, unpublished results). Figure 5 illustrates the molecular model of MAb-chCC49-6P and delineates the six phosphorylation sites fused to the C-terminus of each heavy chain. We have found this structural prediction approach to be particularly useful in the design of phosphorylation sites internal to the protein of interest and in the optimization of the phosphate local milieu to strongly enhance the stability of the radiolabel on the target protein (W. Wu and S. Pestka, unpublished results).

SPECIFIC APPLICATION OF 32 P-LABELED PROTEINS AND MONOCLONAL ANTIBODIES TO TUMOR RADIOIMMUNOTHERAPY

The clinical potential of monoclonal antibodies has begun to be exploited such that MABs specific for human tumor antigens are currently used in the clinical setting, and a large number of novel antibodies are in various stages of human trials. Although MABs can act via complement- or cell-mediated toxicity, some MABs, such as Herceptin and Rituxan, are used directly without radiolabeling or conjugation to cytotoxic agents with sufficient efficacy to gain FDA approval. However, it is believed that the efficacy of these MABs could be markedly improved so that each is being radiolabeled with 131 I or heavy metal isotopes for testing. A number of other radiolabeled antibodies are under development such as a second generation anti-TAG-72 antibody, CC49, and derivatives directed at a human tumor antigen expressed in number of human carcinomas including breast, colon, ovarian, stomach, endometrial, pancreatic, and lung cancers.

A radiolabeled MAB may display greater antitumor efficacy than the unlabeled counterpart because the radiolabel can be

Figure 3. Stability of [32 P]MAB-chCC49CKI in the absence of the preincubation step. Each reaction contained 0.5 mL of human serum, mouse serum, fetal bovine serum, or a solution of bovine serum albumin (5 mg/mL in PBS); 125 mL of 1 M Tris-HCl, pH 7.4, and 15 mL of [32 P]MAB-chCC49CKI (1.1×10^7 cpm), for a total volume of 0.64 mL, and was incubated at 37°C. Aliquots of 20 mL were taken in duplicate over a 48 h period to determine the stability of the [32 P]phosphate attached to the MAB. The percentage of 32 P remaining on the [32 P]MAB-chCC49CKI in sera and buffer at various time points is shown. Experiments were performed as described in the text except that no preincubation step was used to remove loosely attached phosphate after the phosphorylation reaction. Data from Lin et al. (52).

Figure 4. Stability of [32 P]MAB-chCC49KI in various sera. Labile phosphate was removed before use of the labeled monoclonal antibody as described in the text. The percentage of 32 P remaining on the [32 P]MAB-chCC49KI in sera and buffer at various time points is shown. Experiments were performed as described in the text. Data from Lin et al. (49).

lethal to cells within a radius of at least a few cell diameters around the cell to which the antibody has bound. Thus, surrounding tumor cells can be irradiated, even if expression of the tumor antigen on these cells is poor. Furthermore, in contrast to antibody-toxin conjugates, the efficacy of the radiolabel is independent of antibody internalization.

However, the drawbacks of radioiodination or chelation of heavy metal isotopes to MABs include loss of specificity of the antibody for target antigen, and generation of a mixed population of MAB species potentially leading to increased immunogenicity. The remarkable advantages of phosphorylation with ^{32}P are readily apparent from the data presented herein and previously (47–52,82,88). These advantages include virtually quantitative retention of specific immunoreactivity of antibody molecules, a homogeneous population of MAB species imparting lower overall immunogenicity, and a high energy β emission which exhibits excellent tissue absorption to a maximum radius of approximately 10 mm enhancing the percentage of total radiation delivered locally to the tumor.

A further significant advantage of ^{32}P has been documented in recent years. The longer half life of this isotope imparts a greater margin of safety in patients than isotopes such as ^{131}I and many of the heavy metals such as ^{111}In , $^{99\text{m}}\text{Tc}$, ^{90}Y , and ^{186}Re used for monoclonal labeling. Short-lived radionuclides often yield low initial dose rates such that only 3 logs or less of cancer cells can be killed out of the approximately 10 logs required for tumor elimination (17). Longer half-life isotope-bearing MABs deliver greater tumor doses of radiation and have significantly lower adverse effects in bone marrow, i.e., to hematopoietic progenitor cells. Specifically, a comparison of MABs labeled with ^{64}Cu and ^{67}Cu found that the difference in half-life between these two isotopes is responsible for the significantly higher therapeutic index of the

monoclonal bearing ^{67}Cu , the longer-lived isotope (12). In the case of ^{32}P , with the same tumoricidal dose, ^{32}P -labeled antibody results in 30% lower bone marrow exposure to radiation than the same antibody coupled to ^{90}Y (24). An additional concern with chelating agents that are used to attach heavy metal isotopes is the development of antibodies to the chelating agent in almost all patients, resulting in serum sickness in about 40% of patients, a problem avoided with the use of ^{32}P . The *in vitro* success of a ^{32}P -labeled CEA-directed single chain monoclonal in cell culture experiments is encouraging (36). If this efficacy can be extended into human clinical trials, phosphorylated monoclonals may well provide a new weapon in the fight against any number of cancers.

In addition to monoclonal antibodies, this technique can be utilized to enhance the efficacy of other potential protein therapeutic strategies. Because the significant molecular size of MABs often limits their penetrance into tumors, small protein ligands are attractive therapeutic molecules when coupled to toxins or radiolabels. For instance, one well characterized tumor antigen associated with breast and ovarian cancers is the epidermal growth factor (EGF) receptor. EGF receptor peptide antagonists bearing radiophosphate may be exciting candidate molecules for treating such cancers.

CONCLUDING REMARKS

We have described various applications of this procedure for labeling proteins that do not inherently contain phosphorylation sites. However, a variety of proteins have phosphorylation sites that can be phosphorylated directly. For instance, we have used IFN- γ , phosphorylated on the endogenous PKA site quite effectively as a ligand (14,16,22,23,29–31,37,38,40–46,54,55,69–72,79,80). The procedures described in this review provide protein substrates that are conveniently labeled to high radiospecific activity and which are normally as active biologically as the parent molecules. The binding specificity of these proteins is high enough that use of excess competitor to establish nonspecific binding can be largely eliminated. Proteins can be labeled with phosphate or sulfate at slightly modified internal sequences in the protein or on segments added as fusions. This technique also may provide a greater margin of safety for laboratory personnel as well as hospital workers and patients subjected to the hazards of radioimmunotherapy. The therapeutic potential of ^{32}P -labeled proteins is starting to be recognized, and site-specific phosphorylation by kinases shortly will provide novel potential therapeutics.

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Figure 5. Model of MAB-chCC49-6P with six phosphorylation sites. Six phosphorylation sites were attached to the C terminus of the heavy chain of MAB chCC49 as described (49). The light chains are shown in yellow whereas the heavy chains are shown in royal blue. The white regions shown in the space-filling models represent the regions where the protein kinase recognition sites were introduced. The green regions, barely visible, represent the phosphates attached to the serine residues after phosphorylation. Oxygens attached to the phosphates are shown in red.

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