Bacterial Expression of a Kemptide Fusion Protein Facilitates ³²P Labeling of a Humanized, Anti-Carcinoembryonic Antigen (hMN-14) Antibody Fragment

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Abstract

Despite the potential advantages of ³²P over other isotopes for radioimmunotherapy, its development as a therapeutic has been hindered by the difficulty of the labeling chemistry. Recently, a heptapeptide (Kemptide (KPT)) has been chemically conjugated to antibodies, and the conjugates have successfully been labeled with ³²P enzymatically by using bovine protein kinase. By using genetic engineering, we have produced a chimeric (Fab.KPT) containing the Fab’ moiety of the complementarity-determining region-grafted anti-carcinoembryonic antigen-monoclonal antibody, MN14, and a heptapeptide derivative of KPT (Trp-Arg-Arg-Ala-Ser-Leu-Gly). The recombinant protein was expressed in Escherichia coli as a solubile secretory product. The presence of the KPT derivative downstream of the COOH terminus of the hinge region did not impair the binding affinity of the antibody fragment. The Fab.KPT was enzymatically phosphorylated with ³²P by bovine protein kinase, without significant effect on the resultant immunoreactivity; 100% of the ³²P-labeled Fab.KPT was complexed with liquid carcinoembryonic antigen. The ³²P-labeled humanized MN14 Fab.KPT is expected to have longer blood circulation half-life, allowing for an improved therapeutic efficacy in radioimmunotherapy.

Introduction

One of the most important factors that will affect the efficacy of RAIT3 for treatment of cancers is the identification of an optimal radionuclide. The most commonly used isotope is ¹³¹I, which has an 8.05-day physical half-life. It suffers from the disadvantages of having a high abundance medium-energy γ component (86%; 365 keV) associated with its decay, and a propensity to wash out of tumor targets (after localization and subsequent metabolism of iodinated protein) because of dehalogenation (1). As potential replacements for ¹³¹I, a series of alternate nuclides have been suggested and tested (2). Nearly all have half-lives shorter than ¹³¹I, with the 64-h half-life ⁹⁰Y (3) and the 90-h half-life ⁹⁰⁰Re (4) being the most widely investigated. However, the use of later-generation CDR-grafted antibody constructs as targeting vectors is likely to result in radioimmunoconjugates with longer circulation times than murine antibodies (5), and such constructs may be more useful with an isotope having a longer, rather than a shorter, half-life than that ¹³¹I. Independent of antibody form, a number of groups have predicted that the optimal isotope for RAIT will be a relatively long-lived species, to fully take advantage of an extended residence time at the target postlocalization of radioimmunoconjugate (6, 7). Perhaps the most often suggested longer-lived radionuclide for RAIT is the pure β emitter ³²P (Eₘₓ max 1700 keV, 100%; half-life, 14 days), which is inexpensive and readily available in high specific activity. However, relatively little attention has been paid to this isotope, mainly because of the practical difficulties in ³²P radiolabeling of proteins.

Recently, Foxwell et al. (8) used a peptide substrate (KPT) of bovine protein kinase for enzymatic ³²P labeling at the serine residue. The phosphorylated KPTs were then chemically linked to MAb lysine residues by conversion of the NH₂-terminal of the KPT to an iodoacetyl derivative, thiopropionylation of MAb lysine residues, and S-alkylation. Although the method is relatively simple and rapid, it involves labeling of KPTs with the isotope before chemical conjugation to the antibody. Most importantly, the peptide-antibody conjugation was nonsite specific, resulting in antibody conjugates with heterogenous antigen-binding properties. We report here, by using genetic engineering, the construction of a bacterial expression vector for a fusion protein encompassing the Fab fragment of a humanized CEA-specific antibody, hMN-14 (9), fused at its Fab chain COOH terminus with a derivative of the KPT sequence. The effect of such fusion and the enzymatic labeling with ³²P on the antigen-binding properties of the resultant conjugate is evaluated. The ³²P labeling of the fusion protein with bovine protein kinase is also described.

Materials and Methods

Construction of Bacterial Expression Vector for hMN-14 Fab.KPT Fusion Protein. A pUC19-based vector similar to PSV1polyH (10) was used to assemble the bacterial expression vector for hMN-14 Fab fragment. A ribosome-binding site was introduced upstream of a PeB sequence, which was fused in-frame to the NH₂ terminus of the hinge chain variable region, the CH1, and the IgGl hinge region of hMN-14. A termination codon was introduced immediately 3’ to the hinge region-coding sequence. Similarly, a separate ribosome-binding site and PeB sequence were joined in-frame to the gene encoding the κ light chain variable region and κ chain constant region. The heavy and light chain fragments were ligated together as a dicistronic gene, driven by a single inducible LacZ promoter. The structure of the final expression vector, hMN-14pBEV, is shown in Fig. 1. Fusion of the KPT sequence to the hinge region was accomplished by PCR amplification of the partial COOH terminus sequence of CH1 and the hinge-region sequence using the primer pair H-KPT and H5′BX. The PCR-amplified product was then digested with BstXI and BglII and subcloned into the corresponding restriction sites in hMN-14pBEV. The primer H-KPT ('5′-TGC AGA TCT TTA ACC CAA CGA GGC TCT CCT CCA TGG GCA CGG TGG TCT GTG A-3′) is a 55-mer representing the minus strand sequence encoding a KPT variant (nucleotides 13-33) and the last seven COOH-terminal amino acids of the hinge region (nucleotides 34-55). A BglII restriction enzyme digestion site (underlined) was included to facilitate subcloning. The primer H5′BX ('5′-AAG GCC GTG GTG ACC GCC CCC TCC A-3′) is a 22-mer representing the plus strand sequence encoding amino acids 189-195 at the CH1 region (Kabat's numbering; Ref. 11).

Bacterial Expression and Purification of hMN-14 Fab.KPT Fusion Protein. CslPi-purified DNA of hMN-14 KPTpBev bacterial expression vectors were used to transform the E. coli BMH71-18mutS (CLONTECH, Palo Alto, CA). Transformants were grown overnight at 37°C in 2xTY supplemented with 55 mM glucose and 100 μg/ml of ampicillin. The overnight culture was pelleted, washed twice with 2xTY, and resuspended with prewarmed 2xTY in the presence of 1-5 mM isopropyl thiogalactoside. After an induction period of 20-24 h at 37°C, the culture was pelleted and the supernatant was collected for antibody purification with a QuickMab column (Sterogene, Arcadia, CA) by using procedures according to manufacturer’s specifications.
Phosphorylation of hMN-14 Fab.KPT Fusion Protein. The hMN-14 Fab.KPT was subjected to additional purification on a centrifuged Sephadex G-50–80 size-exclusion column (Sigma Chemical Co., St. Louis, MO) by using 0.1 M sodium phosphate (pH 7) as the eluent. The final concentration of the fusion protein was adjusted to ~1 mg/ml. Kinase labeling reaction was accomplished by mixing 6.3 μl of enzyme buffer (250 mM sodium phosphate, 25 mM MgCl₂, and 1.25 mM EGTA) sequentially with 1 μl of [γ-32P]ATP (15.4 μCi), 25 μl of the hMN-14 Fab.KPT solution, and finally 5 μl (25 units) bovine heart protein kinase (Sigma). The reaction mixture was incubated at 37°C, and the progress of the labeling was monitored on a size-exclusion HPLC column (Bio-Sil 250) cluting with 0.2 M sodium phosphate (pH 6.8) at a rate of 1 ml/min. The HPLC eluate was monitored for absorbance at 280 nm and by an in-line radiation detector.

MN-14 Blocking Assay for Immunoreactivity. The antigen-binding properties of hMN-14 Fab.KPT, before and after 32P labeling, were compared to hMN-14 Fab’ fragment prepared from hMN-14 IgG pepsin digestion in a MN-14 blocking assay. ELISA plates precoated with an anti-CEA IgG were treated with anti-CEA MAb MN-5 (Immunomedics, Morris Plains, NJ) and CEA (Calbiochem, San Diego, CA) at a concentration of 50 ng/ml was added, and the plate was incubated for 1 h at 20°C. HRP-conjugated murine MN-14 (Immunomedics), at a concentration of 200 ng/ml, was mixed with varying concentrations of control antibodies, as well as unlabeled or 32P-labeled hMN-14 Fab.KPT. The mixture was incubated for 1 h at 20°C. After washing off the unbound proteins, a substrate solution containing 0.0125% H₂O₂ and 0.004 M o-phenylenediamine dihydrochloride was added to react with the HRP-conjugated MN-14 for 10–20 min at 20°C. The amount of HRP-conjugated MN-14 bound was quantified by measurement of absorbance at 490 nm.

Results

Bacterial Expression of hMN-14 Fab.KPT Fusion Protein. PelB signal peptide sequences were used to transport the heavy and light chains of hMN-14 Fab.KPT to bacterial periplasm for proper folding and intramolecular disulfide linkage formation (9, 12). Immunoreactive components were found both in periplasmic extract (data not shown) and in culture media. Using an ELISA assay, we detected “bacterial secretion” of Fab at a concentration of 2–5 mg/liter of culture media upon isopropylthiogalactoside induction (data not shown). For reasons of simplicity, the Fab.KPT fusion protein was purified directly from the bacterial culture media by using a Sterogene QuickMab antihuman κ chain-affinity column. SDS-PAGE analysis under nonreducing conditions (Fig. 2) indicated that about 90% of the purified Fab.KPT fusion proteins, regardless of the presence of hinge regions, were monomers (Lane 3), running at a position similar to that of the control hMN-14 Fab’. The rest (~10%) appeared as high molecular size species (Lane 3) similar to a dimeric F(ab’)₂ fragment (Lane 1). It should be noted that the amount of dimer formation was subjected to a batch-to-batch variation, depending on the culture condition, induction, and methods of purification. The absence of the Fc portions in the fusion proteins, thus, the Fc-Fc hydrophobic interactions, was likely to be the cause of this preference of monomer to dimer formation of hMN-14 Fab.KPT, leading to random dimerization through disulfide-bridge formation at the hinge region. The heavy chains of the control hMN-14 Fab’ were reduced as doublets (Lane 4), probably as a result of incomplete pepsin digestion at multiple sites. In contrast, both the heavy and light chains of hMN-14 Fab.KPT were reduced to comigrate as a single band at a Mr of ~28,000 (Lane 5), with the disappearance of bands representing the nonreduced dimeric and monomeric forms of hMN-14 Fab.KPT (Lane 3).

HPLC Analysis of Purified hMN-14 Fab.KPT. In a UV₂₅₀–monitored HPLC analysis, two peaks were observed for hMN-14 Fab.KPT fusion protein (Fig. 3a). The major peak (12.2 min), comprising ~90% of the total protein, eluted with a retention time roughly corresponding to that of the control hMN-14 Fab’ (11.8 min; Fig. 3b). However, ~10% of the total fusion protein, constituting the minor peak (10.8 min), eluted with a retention time similar to that of the dimeric control, hMN-14 F(ab’)₂ (10.6 min; Fig. 3c). These results suggested that the purified fusion proteins were pure and were in the form of either monomers (~90%) or dimers (~10%). The slight displacement observed between the fusion proteins and the controls might be due to structural differences; although the sequence for the hinge region and KPT in the fusion protein was well-defined, the exact pepsin digestion sites for the preparation of hMN14 Fab’ and F(ab’)₂ were unknown. Upon labeling the hMN-14 Fab.KPT with 32P, the conjugates were analyzed on size exclusion HPLC column with an in-line radiation detector. As shown in Fig. 3e, peaks corresponding to hMN-14 Fab.KPT (12.1 min) were detected (compare with Fig. 3a). The relative position of the unincorporated [γ-32P]ATP was indicated in Fig. 3d.
represent the dimer-CEA complex. In a gel-mobility assay (Fig. 5), radiolabeled Fab.KPT and its complex with CEA were detected by autoradiography. The uncomplexed fusion protein migrated at a position roughly corresponding to the molecular size of Fab.KPT, M₉ ~30,000 (Fig. 5, Lane 4). This band disappeared when excess CEA was mixed with the fusion protein before loading the gel (Fig. 5, Lane 3); instead, a band with drastically reduced mobility appeared, indicating CEA-Fab.KPT complex formation. It should be noted that the heat-denatured size marker was electrophoresized under reducing SDS-PAGE condition, whereas the Fab.KPT and its complex with CEA were analyzed in a native gel. The size marker only served to give an approximate indication to the rate of migration of the Fab.KPT before and after complexing with CEA.

Mobility Shift Assay for CEA Binding by ³²P-labeled hMN-14 Fab.KPT. The ability of the ³²P-labeled fusion protein to complex with liquid CEA was assessed by either HPLC or native gel-mobility assay. In a radio-HPLC analysis, the uncomplexed fusion proteins appeared mainly as monomers (major peak) with 10% of dimers constituting the minor peak (Fig. 4a). Upon complexing with excess CEA, both peaks disappeared and shifted to a position corresponding to the higher molecular sized CEA-Fab.KPT complex (Fig. 4b), suggesting that both the monomers and dimers of the fusion protein were immunoreactive. The shoulder peak observed in Fig. 4b should represent the dimer-CEA complex. In a gel-mobility assay (Fig. 5), radiolabeled Fab.KPT and its complex with CEA were detected by autoradiography. The uncomplexed fusion protein migrated at a position roughly corresponding to the molecular size of Fab.KPT, M₉ ~30,000 (Fig. 5, Lane 4). This band disappeared when excess CEA was mixed with the fusion protein before loading the gel (Fig. 5, Lane 3); instead, a band with drastically reduced mobility appeared, indicating CEA-Fab.KPT complex formation. It should be noted that the heat-denatured size marker was electrophoresized under reducing SDS-PAGE condition, whereas the Fab.KPT and its complex with CEA were analyzed in a native gel. The size marker only served to give an approximate indication to the rate of migration of the Fab.KPT before and after complexing with CEA.

Fig. 3. UV (λ₂₈₀)-HPLC analysis of hMN-14 Fab.KPT fusion protein (a), hMN-14 Fab' (b), and Fab' (c) prepared from pepsin digestion of whole IgG; Radio-HPLC analysis of free [γ-³²P]ATP (d) and ³²P-labeled hMN-14 Fab.KPT fusion protein (e). AU, absorbance unit.

Fig. 4. Radio-HPLC analysis of hMN-14 ³²P-labeled Fab.KPT fusion protein (a) and hMN-14 ³²P-labeled Fab.KPT complexed with 80-fold molar excess of CEA for 10 min at room temperature (b).

Fig. 5. Polyacrylamide gel analysis (4%–20%) on ³²P-labeled Fab.KPT fusion protein before (CEA−) and after (CEA+) complexing with 10-fold excess of liquid CEA. kD, molecular weight in thousands; HMW, high molecular weight size markers; LMW, low molecular weight size markers.

Fig. 6. MN-14 blocking assay comparing the CEA-binding affinities between control hMN-14 Fab' (d) fragment prepared from pepsin digestion of hMN-14 IgG (b) and unlabeled (b) and ³²P-labeled (c) hMN-14 Fab.KPT fusion proteins. OD (optical density), absorption.
Effect of KPT Fusion and $^{32}$P Labeling on Immunoreactivity.

The antigen (CEA)-binding affinities of the fusion hMN-14 Fab.KPT before and after phosphorylation were compared to that of hMN-14 F(ab)$_2$ fragments prepared from proteolytic digestion. All three antibody fragments competed equally well with a peroxidase-conjugated murine MN-14 for binding to CEA-coated ELISA plates (Fig. 6), indicating that neither the KPT fusion nor the kinase-labeling reaction would have an adverse effect on the immunoreactivity of the resultant antibody fragments.

Discussion

The generation of Fab.KPT fusion proteins by methods of genetic engineering ensures the production of homogenous species with a defined attachment site for KPT. Bacterial signal sequences such as pelB, alkaline phosphatase, or outer membrane protein were included at the NH$_2$ terminus of the Fabs to facilitate their transportation to the periplasm for proper folding and disulfide-linkage formation (12, 13). Leakage of these periplasmic Fabs into the culture media was in effect resembling the process of mammalian secretion and thus allowing their purification directly from culture media (10). Although prokaryotic expression of Fab or Fab-fusion fragments is simple, rapid (days for bacterial transformation versus weeks for mammalian cell transfection), and economical, it is not without limitations. By including the hinge region in the KPT fusion protein (Fig. 1), we hoped to provide flexibility, possibly required for kinase phosphorylation of the KPT, and to encourage interchain disulfide linkages (total of three cysteines for potential linkages) for the generation of divalent hMN-14 Fab.KPT fusion proteins. Although a small percentage (~10%) of the fusion proteins were divergent, the majority remained as monovalent species (Figs. 2 and 3). Attempts to directly label the monovalent species at the hinge-region cysteine residues with $^{99m}$Tc failed; however, when reduced, these sulfhydryl groups could be partially recovered for $^{99m}$Tc labeling. Oxidation of the sulfhydryl groups, which accounted for the unavailability of free SH groups for direct $^{99m}$Tc labeling might either occur at the periplasm or during the purification process. Nevertheless, the results indicated that additional CH domain interactions are required for proper positioning for interchain hinge-region disulfide linkages. However, bacterial expression of whole IgGs to confirm the suggestion has not been successful.

Unlike eukaryotic cells, bacteria lack the machinery for protein glycosylation. For antibodies the immunoreactivities of which were dependent on V-region glycosylations, such as the antidextran MAbs 14.6b.1, 5.54, and 19.22.1 and the antidansyl antibody from hybridoma 27.44 (14, 15), their expression in bacteria would compromise their antigen-binding properties. In contrast, for antibodies the V-region glycosylation of which would result in no effects on (16, 17) or reduction of (18) immunoreactivities, a bacterial expression system would then be an appropriate or even a preferred choice for their Fab production.

Although $^{32}$P labeling of Fab.KPT by protein kinase provides a fast, simple, and efficient way of radiolabeling, the stability of the incorporated $^{32}$P is a main concern for the usefulness of the method, especially when the possibility of dephosphorylation by serum phosphatase is considered. $^{32}$P as free phosphate is well known to rapidly incorporate as free phosphate is well known to rapidly localize to bone, which would result in unacceptable toxicity. Although the $^{32}$P-labeled hMN-14 Fab.KPT remained stable upon storage in PBS, preliminary studies suggested that about 5–20% of $^{32}$P would be dissociated from the fusion protein incubated in human serum at 37°C for 24 h, depending on the serum sources. A thorough serum stability test over a longer period of time has yet to be established.

By varying the KPT sequences, a variety of different phosphopeptides with improved stability to in vivo phosphatases in mice were generated (19). Most significantly, the replacement of the serine by threonine in the KPT sequence, although it compromises the ease of phosphorylation, resulted in an improved resistance to dephosphorylation. Peptide sequences with optimized labeling efficiency and resistance to dephosphorylation are being developed. Incorporation of these peptides with optimized sequences to Fab as fusion proteins should facilitate their stable $^{32}$P labelings. Alternatively, serum stability and the ease of enzymatic $^{32}$P incorporation can be improved by using different phosphorylation enzymes and substrate sequences. For example, enzymes such as mitogen-activated protein kinases and protein tyrosine kinases recognizing different peptide substrates and found abundantly in intracellular signal transduction pathways can be used instead of bovine protein kinases (20–23). Mitogen-activated protein kinase is a serine/threonine kinase that recognizes and phosphorylates the threonine residue (underlined) in the sequence TPRTPPP found in myelin basic protein (20). A number of cellular proteins with protein tyrosine kinase activities were reported (21–23). To name a few, they include p53/S6, p56, p54, p55, p57, etc. These proteins recognize and phosphorylate at the tyrosine residues of a variety of specific sequences.

In conclusion, we have demonstrated that the successful construction and expression of a Fab.KPT fusion protein have facilitated the difficult task of $^{32}$P labeling by a quick and easy enzymatic approach, without the need for subsequent chemical manipulation. The abundance of natural enzyme-peptide substrate pairs for phosphorylation has provided a variety of possibilities for the optimization of the fusion, phosphorylation, and $^{32}$P stability for achieving maximum serum stability and therapeutic efficacy.

References


$^{32}$P LABELING OF AN ANTIBODY FRAGMENT KEMPTIDE FUSION PROTEIN


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