

Molecular Position of Radiolabels and Its Impact on Functional Integrity of Proteins

Therapy control is one of the main areas of interest during the follow-up of cancer patients. This information is currently acquired by a series of conventional investigations, including morphologic changes, symptomatic responses, and clinical chemistry. Techniques such as radiography, CT, sonography, and MRI are imaging techniques that cover the extent and morphology of tumor masses and, to some degree, alterations in tumor perfusion. In contrast, nuclear medicine has at its disposal a series of radiopharmaceuticals that are successfully used for the assessment of the therapy response. Among these agents,

See page 1546

^{18}F -FDG acts as a surrogate marker showing the influence of, for example, cytostatic drugs on the glucose transport and hexokinase activity of tumors. A decrease in tracer accumulation is expected to indicate a treatment response and a better survival. However, interpretation of the signal may be complicated by inflammatory reactions, such as those that can occur early after radiation therapy. Furthermore, the tumor response is shown through a negative signal—a decrease in tracer uptake that may be problematic in cases of moderate accumulation

before therapy. In this respect, tracers providing a positive signal are more desirable.

Eight years ago, $^{99\text{m}}\text{Tc}$ -labeled annexin V was presented for the first time as a new agent capable of imaging cell death *in vivo*. Apoptosis was visualized in several model systems, including myocardial infarction and Fas antibody-treated liver and tumors after cytostatic drug application (1,2). The use of $^{99\text{m}}\text{Tc}$ -labeled annexin V as an *in vivo* imaging indicator for apoptosis was based on results obtained with a previously developed annexin V–fluorescent dye conjugate used to show apoptotic cells under a microscope (3). Annexin V was first isolated from human placenta (4) and demonstrated an anticoagulant effect by inhibiting, among other factors, prothrombinase activity. This effect was attributed to the displacement of coagulation factors from the phospholipid membrane (5).

Annexins are ubiquitous homologous proteins that bind to phospholipids in the presence of calcium. Annexin V, formerly also known as placental anticoagulant protein or vascular anticoagulant protein (5), binds together with Ca^{2+} on externalized phosphatidylserine (PS) present on cell membranes early after the onset of apoptosis. Under normal conditions, nearly 100% of PS resides on the interior leaflet of the bilayer cell membrane, where it functions as an anchor for annexin I, which is involved in transmembrane signaling and internalization processes (6). The activation of effector caspases 3, 6, and 7, which are induced after either intrinsic or extrinsic stimuli, results in the proteolytic inactivation of proteins that maintain, under normal condi-

tions, the asymmetric distribution of lipids within the cell membrane. Among these proteins are several types of translocase enzymes, including a ubiquitous Mg^{2+} -adenosine triphosphate (ATP)-dependent aminophospholipid flippase that selectively catalyzes the inward transport of PS and phosphatidylethanolamine, an ATP-dependent floppase that moves phospholipids outward with little head group specificity, and a Ca^{2+} -dependent scramblase that destroys the bilayer asymmetry (7). ATP depletion attributable to energy-demanding apoptotic process also may hamper the maintenance of the asymmetric lipid distribution.

The high affinity of annexin V for cells with exposed PS exhibiting a dissociation constant of less than 10^{-10} mol/L (8) is the basis for detecting apoptosis *in vivo*. What is the structural background for the strong annexin V–PS interaction? It is known from x-ray structure analyses that this kind of binding functions through the cocomplexation of Ca^{2+} with the carboxyl-phospholipid head group in collaboration with AB, AB', and DE Ca^{2+} -binding sites. These binding sites are located in each of the 4 tandemly repeated segments of annexin V. Figure 1 shows the interaction of one of these segments (domain III) with glycerophosphoserine (truncated PS) on Ca^{2+} -annexin V-binding sites (9). Primary AB and secondary AB' Ca^{2+} -binding sites are located within the loop between the A and B helices of each domain. They also are called type II and type III Ca^{2+} -binding sites, respectively.

It should be noted that the 3 complexation sites—AB, AB', and DE—imply differentially coordinated calcium

Received Apr. 25, 2006; revision accepted May 8, 2006.

For correspondence or reprints contact: Michael Eisenhut, PhD, Department of Radiopharmaceutical Chemistry, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany.

E-mail: m.eisenhut@dkfz.de

COPYRIGHT © 2006 by the Society of Nuclear Medicine, Inc.

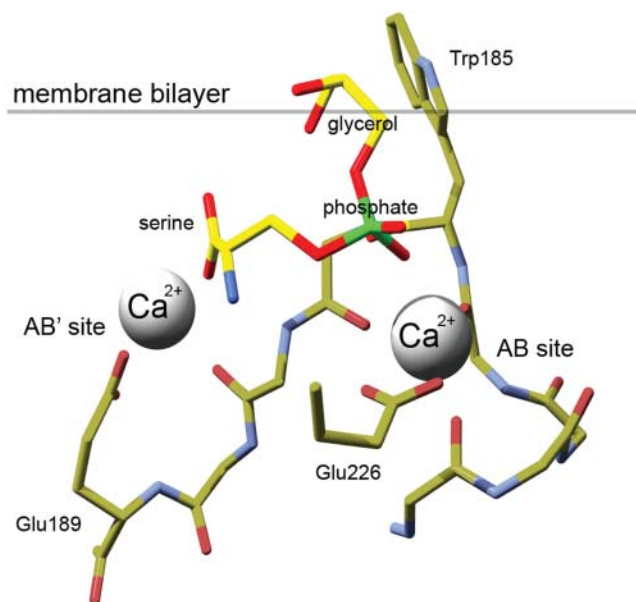


FIGURE 1. Structural alignment of glycerophosphoserine (bright colors) in AB and AB' Ca^{2+} -binding sites in third domain of annexin V (muted colors). Amino and amide nitrogens are in blue, oxygen and hydroxyls are in red, and phosphorous is in green (modified structure (9); courtesy of Barbara A. Seaton, written personal communication, April 13, 2006).

cations with different stabilities: site AB > site AB' > site DE. The second complex entity, PS, coordinates Ca^{2+} in the AB' site with the carboxyl function of serine and with the negatively charged phosphate oxygen in the AB site. In addition, the protonated α -amino group of serine appears to stabilize and interconnect the two Ca^{2+} sites through hydrogen bonds with neighboring main chain amino acids (9).

The interaction of annexin V with PS through jointly used Ca^{2+} is, however, not solely responsible for the strong binding affinity. Multivalent binding must be considered because Ca^{2+} forms peptide complexes with a dissociation constant K_d of only 10^{-4} – 10^{-5} mol/L. In combination with PS, this value may decrease to some

degree because of the bidenticity of PS for Ca^{2+} . However, to meet the above-mentioned small dissociation constant, 2 or more domains must be involved in annexin V– Ca^{2+} –PS head group interactions. These considerations assume that enough Ca^{2+} is present. The Ca^{2+} dependence was validated by titration in the presence of annexin V binding at low membrane occupancy, showing the strong cooperativity of this interaction with respect to Ca^{2+} (10). Up to 12 Ca^{2+} atoms theoretically can be complexed by 3 binding sites—AB, AB', and DE—in each of the domains. As many as 10 Ca^{2+} atoms have been located in rat annexin V crystal structures—all binding at the membrane-facing surface of the protein (9).

These interactions are potentially delicate with respect to disturbing substituents such as chelators and radiometals attached to the lysine residues of annexin V. This is especially true when lysine belongs to the sequence that coordinates Ca^{2+} and interacts with PS. Table 1 shows the sequence homology in the Ca^{2+} -binding loops of annexin V (11). Amino acids in bold type contribute main-chain carbonyl oxygen ligands to the AB Ca^{2+} ion. These sequences contain 4 of the 22 lysine residues (K) present in the protein. Lysine residues in the interacting area are potentially endangered for amine-directed radioligands.

An interesting investigation of site-specific radiolabeling is reported by Tait et al. on pages 1546–1553 of this issue of *The Journal of Nuclear Medicine* (12). They present a systematic investigation of the influence of amine-directed tags on annexin V binding. The modifications included conjugation reactions of activated esters of hydrazinonicotinic acid, mercaptoacetyltriglycine, and biotin as well as fluorescein isothiocyanate. These conjugates, labeled with $^{99\text{m}}\text{Tc}$, were compared with annexin V-128 carrying a site-specific $^{99\text{m}}\text{Tc}$ label positioned at the N-terminal end. This was made possible by implementing the amino acid sequence Ala-Gly-Gly-Cys-Gly-His by recombinant techniques. N-terminal $^{99\text{m}}\text{Tc}$ was clearly shown to be superior to randomly distributed lysine tags. This work represents a continuation of earlier efforts investigating recombinant techniques to introduce chelating peptide sequences at the N terminus (13) and to improve the imaging quality of annexin V (14).

TABLE 1
Sequence Homology in AB Ca^{2+} -Binding Site Loops of Annexin V*

Domain	Residues	Sequence								
I	26–31, 72	M		K	G	L	G	T	E
II	98–103, 142	L		K	G	A	G	T	D
III	181–187, 226	G	E	L	K	W	G	T	E
IV	257–262, 301	M		K	G	A	G	T	D

*Data are from Seaton et al. (11).

The 36-kDa annexin V is obviously more sensitive to lysine-directed substituents than are monoclonal antibodies (~150 kDa). This result might be expected on the basis of knowledge of the amino acid sequences shown in Table 1. Amine-directed substituents carrying complexed ^{99m}Tc generally do not compromise the biologic characteristics of proteins, as shown with a series of monoclonal antibodies conjugated with up to 4 heterobifunctional ligands (Bis(aminoethanethiol) type) per protein molecule without a loss of immunoreactivity (15). Binding began to decrease above that conjugation ratio. Although the number of complexed ligands in relation to the molecular protein mass were about the same in the antibodies (4/150 kDa) as in the modified annexins (1/36 kDa), this result surely was favored by the lack of lysines in the complementarity-determining regions of the antibodies. In another study (16), the incorporation of radioiodine with various specific activities into heavy and light chains of 13 monoclonal antibodies was studied. Radioiodine is less space demanding than the technetium complexes mentioned above, but this substituent may change the hydrophobic nature of the variable regions if tyrosine residues are present. In that study, several antibodies proved stable while others did not, even at a low dose (16).

One of the main lessons that can be learned from the investigation of Tait et al. (12) is that knowledge about the

amino acid composition of the binding site is an indispensable condition. Site-specific labeling is the solution of choice, especially when recombinant techniques are available. One of the other techniques that uses site-directed labeling is the application of C-terminal histidine tags on recombinant antibodies, which can be used to bind ^{99m}Tc through the exchange of water from the $[\text{}^{99m}\text{Tc}(\text{CO})_3(\text{H}_2\text{O})_3]^+$ complex (17).

Michael Eisenhut

German Cancer Research Center
Heidelberg, Germany

Uwe Haberkorn

University of Heidelberg
Heidelberg, Germany

REFERENCES

- Blankenberg FG, Katsikis PD, Tait JF, et al. Increased localization of ^{99m}Tc -hydrazinonicotinamide (HYNIC)-labeled annexin V lipocortin in lymphatic tissue of animals treated with dexamethasone [abstract]. *J Nucl Med.* 1997;38(suppl):268P.
- Blankenberg FG, Katsikis PD, Tait JF, et al. In vivo detection and imaging of phosphatidylserine expression during programmed cell death. *Proc Natl Acad Sci U S A.* 1998;95:6349–6354.
- Koopman G, Reutelingsperger CP, Kuijten GA, Keehnen RM, Pals ST, van Oers MH. Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood.* 1994;84:1415–1420.
- Bohn H, Kraus W. Isolation and characterization of a new placenta specific protein (PP10). *Arch Gynecol.* 1979;227:125–134.
- Reutelingsperger CP, Hornstra G, Hemker HC. Isolation and partial purification of a novel anticoagulant from arteries of human umbilical cord. *Eur J Biochem.* 1985;151:625–629.
- Rescher U, Zobiack N, Gerke V. Intact $\text{Ca}(2+)$ -binding sites are required for targeting of annexin I to endosomal membranes in living HeLa cells. *J Cell Sci.* 2000;113:3931–3938.
- Boon JM, Smith BD. Facilitated phosphatidylcholine flip-flop across erythrocyte membranes using low molecular weight synthetic translocases. *J Am Chem Soc.* 2001;123:6221–6226.
- Tait JF, Gibson D, Fujikawa K. Phospholipid binding properties of human placental anticoagulant protein-I, a member of the lipocortin family. *J Biol Chem.* 1989;264:7944–7949.
- Swaarjo MA, Concha NO, Kaetzel MA, Dedman JR, Seaton BA. $\text{Ca}(2+)$ -Bridging mechanism and phospholipid head group recognition in the membrane-binding protein annexin V. *Nat Struct Biol.* 1995;2:968–974.
- Tait JF, Gibson DF, Smith C. Measurement of the affinity and cooperativity of annexin V-membrane binding under conditions of low membrane occupancy. *Anal Biochem.* 2004;329:112–119.
- Seaton BA. Annexin V molecular structure, ligand binding and biological function. In: Seaton BA, ed. *Annexins: Molecular Structure to Cellular Function.* Austin, TX: R.G. Landes Co.; 1996:15–29.
- Tait JF, Smith C, Levashova Z, Patel B, Blankenberg FG, Vanderheyden J-L. Improved detection of cell death in vivo with annexin V radiolabeled by site-specific methods. *J Nucl Med.* 2006;47:1546–1553.
- Tait JF, Brown DS, Gibson DF, Blankenberg FG, Strauss HW. Development and characterization of annexin V mutants with endogenous chelation sites for ^{99m}Tc . *Bioconjug Chem.* 2000;11:918–925.
- Tait JF, Smith C, Blankenberg FG. Structural requirements for in vivo detection of cell death with ^{99m}Tc -annexin V. *J Nucl Med.* 2005;46:807–815.
- Eisenhut M, Lehmann WD, Becker W, et al. Bifunctional NHS-BAT ester for antibody conjugation and stable ^{99m}Tc labeling: conjugation chemistry, immunoreactivity and kit formulation. *J Nucl Med.* 1996;37:362–370.
- Matzku S, Kirchg bner H, Nissen M. Iodination of monoclonal IgG antibodies at a sub-stoichiometric level: immunoreactivity changes related to the site of iodine incorporation. *Int J Rad Appl Instrum B.* 1987;14:451–457.
- Waibel R, Alberto R, Willuda J, et al. Stable one-step technetium-99m labelling of His-tagged recombinant proteins with a novel $\text{Tc}(\text{I})$ -carbonyl complex. *Nat Biotechnol.* 1999;17:897–901.