Optimal Modification of Annexin V with Fluorescent Dyes

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The many uses of chemically modified annexin Vs necessitate an understanding of the optimal degree of modification and modification sites of the protein. When reacted with the N*-hydroxysuccinimide ester of Cy5.5, annexin V with one modification per mole of protein retained its affinity for phosphatidylserine of apoptotic cells, whereas modification with two dyes per mole of protein caused a complete loss of activity. A tryptic digest LC/MS method was used to identify the modification sites as either of two closely spaced lysine residues, in position 286 or 290. The crystal structure indicated the location of these lysines was distal to the phosphatidylserine binding sites on annexin V. These results can be used to develop active or inactive fluorescent control annexin V proteins and to suggest strategies for attaining higher levels of modification with retention of bioactivity.*

Introduction

Programmed cell death (apoptosis) is an essential feature of normal tissue homeostasis and tissue differentiation.[1] There are a variety of markers that can be used to identify apoptotic cells including morphologicial criteria, DNA fragmentation, and assays for caspase activity.[2, 3] However the identification of intact apoptotic cells is often accomplished by using annexin V, which binds to phosphatidylserine (PS), a lipid normally facing the cytoplasm which flips and faces the extracellular fluid early in apoptosis.[4, 5] This has led to the synthesis of a number of chemically modified, annexin V-based probes. A hydrazinonicotinamide(Hy-Nic)-modified annexin is used for technetium labeling,[6–11] while *N*-succinimidyl 3-(2-pyridylthio)propionate(SPDP)- or *S*-acetylthioactetate(SATA)-modified annexins are used to synthesize magnetic annexin Vs. Magnetic annexin Vs are used to magnetically manipulate apoptotic cells or as MR contrast agents for imaging apoptosis.[12, 13] Annexin V labeled with fluorescein isothiocyanate (FITC) is widely used for identifying apoptotic cells by fluorescence-activated cell sorting (FACS) analysis,[14, 15] while a Cy5.5-labeled annexin V has been used for near-infrared fluorescent optical imaging.[16, 17]

In order to obtain optimal probes for these many applications, it is necessary to understand how to modify annexin V to the maximum extent possible whilst insuring that modification does not compromise bioactivity. In spite of the many uses of modified annexin Vs, there are no reported studies of annexin V modification that indicate an optimal degree of modification or where modifications to the protein have occurred. Here, we examine the bioactivity of annexin V modified to different extents with different fluorochromes, and we determine the modification sites by using the *N*-hydroxysuccinimide ester of Cy5.5. We employed a tryptic digest/LC-MS method widely used for the identification of proteins to elucidate sites of annexin V modification. We found the binding of annexin V to apoptotic cells was sensitive to the modification and detected modification on specific lysines with a conjugate modified by the attachment of 1.1 Cy5.5 dyes per mole of protein. These studies allow for production of improved active forms of annexin V and suggest new strategies for modifying the protein.

Results and Discussion

The binding of dye-modified annexin Vs to apoptotic cells was assessed by their ability to displace or decrease $FITC_{1.6}$–annexin binding in a FACS-based assay (Figure 1). FACS permits quantitation of $FITC_{1.6}$–annexin binding to cells, and its displacement by Cy5.5–annexin V or Cy7–annexin Vs, which are spectrally distinct from fluorescein. For example, $Cy5.5_{2.4}$–annexin V had a relative binding of 0.98 relative to a reference value of 1.0 for no added annexin V. Therefore, $Cy5.5_{2.4}$–annexin V is a fluorescent but inactive form of annexin V. On the other hand, $Cy5.5_{1.1}$–annexin V had a relative binding of 0.32, compared to a value of 0.17 for unlabeled annexin V. Figure 1 also illustrates the inactivation produced by modification with the *N*-hydroxysuccinimide ester of Cy7. To obtain comparable data for $FITC_{1.6}$–annexin V, the ability of $FITC_{1.6}$–annexin V to displace $Cy5.5_{1.1}$–annexin V was obtained. Optimal modification of annexin V occurred at between 0.8 and 1.6 lysine residues per mole of protein, since lower levels of modification will produce mixtures of unmodified and modified annexin V, and higher levels were associated with the loss of binding activity.

$Cy5.5_{1.1}$–annexin V was submitted to tryptic digestion HPLC/mass spectrometry to determine the sites of modification by

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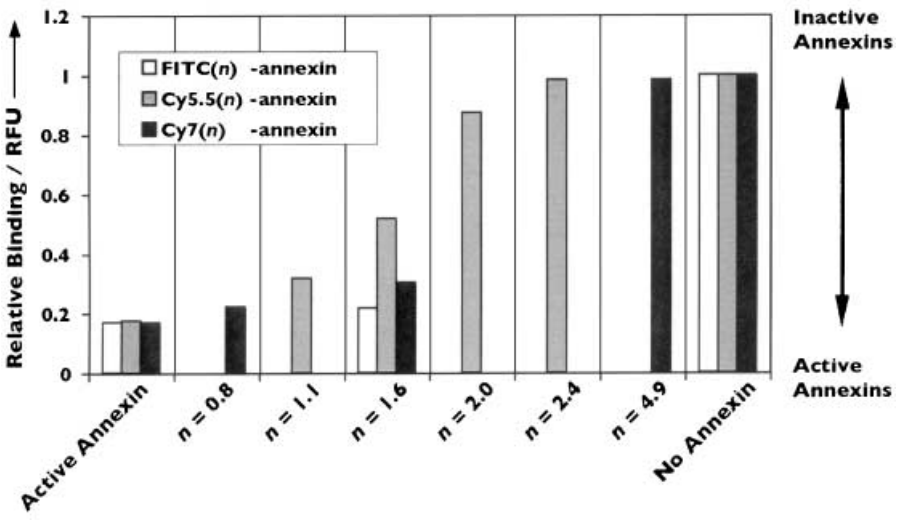

Figure 1. *Binding of dye – annexin V conjugates to camptothecin treated Jurkat T cells. Active Cy5.5 – annexin V or active Cy7 – annexin V displaced cell-bound $FITC_{1.6}$ – annexin V, thereby decreasing the median fluorescence and yielding a low relative binding value. Inactive Cy5.5 – annexin V or inactive Cy7 could not displace $FITC_{1.6}$ – annexin V and yielded a high median fluorescence or relative binding value. To obtain comparable data for $FITC_{1.6}$ – annexin V, the ability of $FITC_{1.6}$ – annexin V to displace $Cy5.5_{1.1}$ – annexin V was obtained. Relative binding was obtained by normalizing to the peak median fluorescence of apoptotic cells by FACS.*

prominently modified lysine residues. These lysine residues lie distal to the multiple calcium binding sites that interact with PS (see the gray plane in Figure 2).[18]

$Cy5.5_{1.1}$ – annexin V can be used with ease in conjunction with FITC-labeled probes because there is no spectral overlap between the two dyes. We compared the kinetics of exposure to PS and development of caspase activity after Jurkat T cells were exposed to apoptosis-inducing camptothecin. Caspase activity was determined by the uptake of the pan caspase probe FITC-VAD-FMK (fluorescein isothiocyanate-valyl-alanyl-aspartyl-fluoromethylketone). Figure 3 A shows the development of caspase-positive (fluorescein FL-1 channel) and phosphatidylserine-positive (Cy5.5 FL-4 channel) cells at different times after treatment with camptothecin. Data from Figure 3 A, together with additional time points, are shown in Figure 3 B. Camptothecin results in increasing percentages of both caspase-negative/annexin V-positive (necrotic cells, 31.1% at 15 h) and caspase-positive/annexin V-positive cells (apoptotic cells, 49.5% at 15 h). It

Cy5.5. The molecular weights of selected peptides observed ("MW obs" in Table 1) were compared with the weights of peptides predicted for annexin V ("MW pred" in Table 1). Amino acid numbering is for the 320 amino acid form of annexin V with a methionine residue at position 1. A single peptide with the additional mass expected for a Cy5.5-modified peptide was obtained (2880.2 Da). The molecular weight of the Cy5.5-modified peptide was calculated by adding the molecular weight of Cy5.5 in the acid form (918.06 for $C_{41}H_{45}N_2O_{14}S_4^+$, calculated with ChemDraw from Cambridgesoft) to the weight of peptide KEFRKNFATSLYSMIK (1980.33) and subtracting one mole of water (18.02) to yield 2880.4 Da. However, the 286 – 301 sequence of the peptide (KEFRKNFATSLYSMIK) was consistent with the modification of either of two lysine residues, in position 286 or 290 (these are underlined in Table 1). Since modification of lysine by the addition of the large Cy5.5 adduct would make that lysine resistant to trypsin, modification of the C-terminal Lys301 can be excluded. The incomplete modification of lysine residues at positions 286 and 290 can be inferred from the presence of peptides 287 – 301 and 291 – 301, which also require unmodified lysine residues at positions 286 and 290 to permit trypsin cleavage. For $Cy5.5_{1.1}$ – annexin V, Cy5.5 on Lys286 or Lys290 was detectable, and these are one (or two) of the most

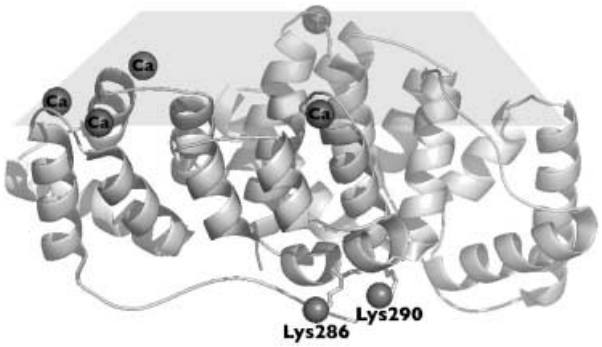

Figure 2. *Structure of annexin V and sites of modification by Cy5.5. The Cy5.5 dyes are coupled to the primary amine of Lys268 or Lys290. The calcium atoms (gray balls) define the membrane-binding site of annexin V (gray plane). Both modification sites are located well outside the membrane binding area.*

Table 1. *Mass spectrometry of peptides from tryptic digest of $Cy5.5_{1.1}$ – annexin V.*

Amino acid sequence	Amino acids	M_W obs/M_W pred	Comment
KEFRKNFATSLYS*M*IK*	286 – 301	2880.2/2880.4 unmodified Lys 301	modified lys 286 or 290
EFRKNFATSLYS*M*IK	287 – 301	1849.5/1852.1	Unmodified lys 286 and 301.
NFATSLYS*M*IK	291 – 301	1290.8/1291.5	unmodified lys 290 and 301

*Two underlined Ks, 286 and 290, are possible sites of Cy5.5 modification, one of which is modified. Methionines (in italics) are methionine sulfoxide residues. Amino acids 277 – 301 are SEIDLFNIRKEFRKNFATSLYS*M*IK.

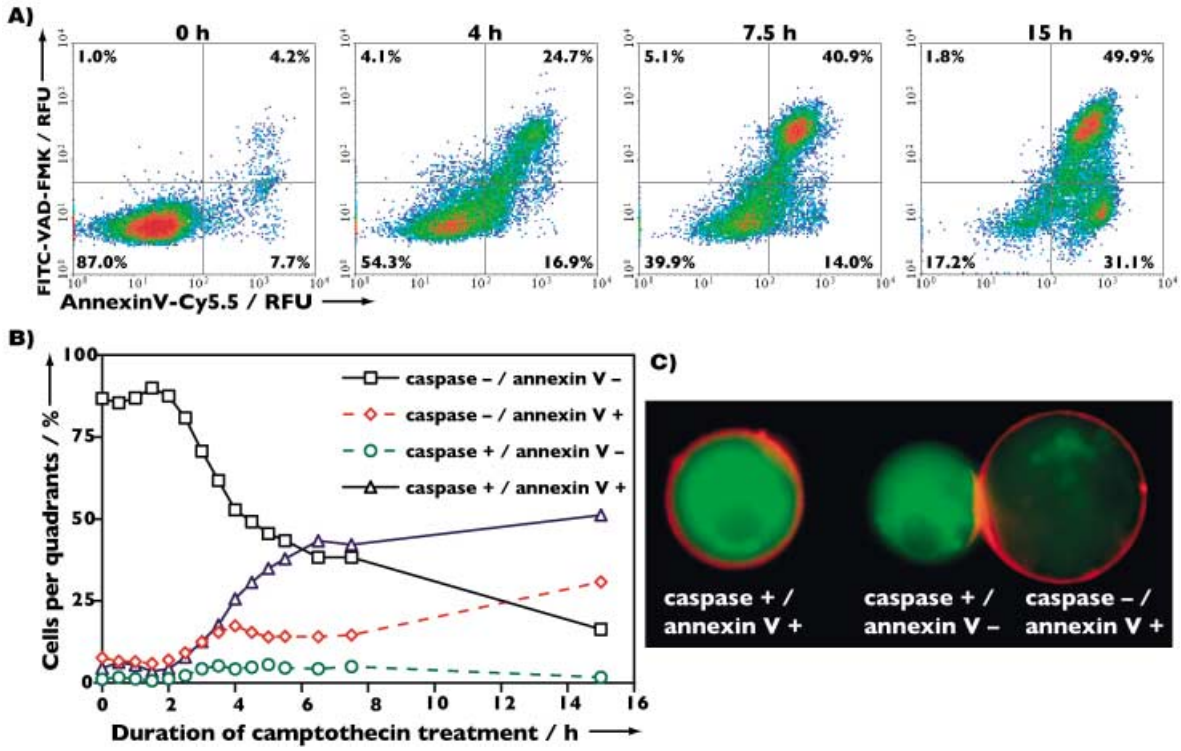

Figure 3. *Time course of Cy5.5$_{1.1}$ – annexin V binding and caspase activation after Jurkat T cells were treated with camptothecin. A) FACS analyses at selected times after induction of apoptosis (0, 4, 7.5, and 15 h); RFU stands for relative fluorescent unit. FL1 channel is from the caspase probe FITC-VAD-FMK. FL4 channel is from Cy5.5$_{1.1}$ – annexin V. B) Percentages of cells in each of four quadrants in (A) are shown along with additional time points. Cells that are caspase-negative/annexin V-positive are evident after 2 – 4 h of treatment. C) Fluorescent micrographs of representative cells shown in (A) and (B). The caspase probe is green and cytoplasmic while the annexin V is red and membrane-localized. A rare caspase-positive/annexin V-negative cell (found in the upper left quadrant of (A) touches more numerous caspase-negative/annexin V-positive cells in the (lower right quadrant) in the micrograph.*

appears that treatment with camptothecin results in the development of caspase-positive and annexin V-binding cells with a similar time course.

Conclusion

In conclusion, annexin V can be modified by reactive amine dyes with retention of its binding to apoptotic cells provided that care is taken to insure that the degree of modification is kept between 0.8 and 1.6 modifications per mole of protein. With 1.1 moles of Cy5.5 per mole of protein, labeling occurs at multiple lysine residues but was detectable at either of two lysine residues distal from the phospholipid-binding plane of the protein. Owing to the sensitivity of annexin V to modification, more highly modified forms might best be accomplished by using a single-point polymer attachment strategy[19] or genetically modified annexin V.[20] In addition, inactive, "overlabled" forms of dye – annexin Vs (Cy5.5 and Cy7) provide ideal controls for unspecific probe binding or accumulation, since many of their physical properties are similar to the dye – annexin Vs which bind apoptotic cells.

Experimental Section

Labeling of annexin V with fluorescent dyes: Annexin V was obtained from Theseus Imaging Corp. and was identical to that used clinically. Annexin V migrated as a single band of 36 kDa by SDS PAGE which confirmed its purity (data not shown). Annexin V (3.0 mg mL^{-1}) was dialyzed against bicarbonate (0.1 M, pH 8.0) for reaction with fluorochromes. To synthesize dye – annexin – Cy5.5 conjugates with a range of dye-to-protein ratios, a vial of the *N*-hydroxysuccinimide ester of Cy5.5 (200 – 300 μg, Amersham – Pharmacia) was solubilized with DMSO (7 μL) and 1, 2, or 4 μL of this solution was added to annexin V (30 μg) to give a final volume of 20 μL. The reaction tubes were incubated for 1.5 h at room temperature. After adding phosphate-buffered saline (PBS; pH 7.4, 30 μL), protein was separated from unreacted dye by two successive spin separations by using 1-mL Biospin P6 columns equilibrated with PBS (pH 7.4, Bio-Rad). The reaction of annexin V (1 mg) with 2 vials of Cy5.5 (400 – 600 μg of dye) resulted in about 1 dye Cy5.5 per mole of protein (with batch to batch variations). The ratio of dye to protein was calculated spectrophotometrically (Cy5.5 $E_{678} = 250\,000$, Cy7 $E_{747} = 200\,000$, fluorescein $E_{493} = 73\,000$). Protein was determined by the BCA method (Bio-Rad). Conjugates are referred to as dye$_n$ – annexin V where n is the moles of dye per mole of protein.

Activity of dye – annexin V conjugates: Jurkat T cells (Clone E6 – 1, ATCC number TIB-152) were grown in RPMI 1640 medium (#30-2001, ATCC) with 10% fetal bovine serum (#30 – 2021, ATCC), which was changed every two or three days. Apoptosis was induced by the

addition of 7 μL camptothecin (1 mM in DMSO) per mL culture medium and incubating for 5–6 h at 37 °C. Induction of apoptosis was verified by staining with propidium iodide and FITC–annexin V by using a calcium-containing binding buffer (1.8 mM $CaCl_2$, 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, pH 7.4). The cells were analyzed with a FACS-Calibur cytometer (Becton Dickinson) according the manufacturer's instructions.

Displacement assay for binding activity: Camptothecin-treated Jurkat T cells were incubated with a 10-fold protein excess (1.0 μg of inhibitor dye–annexin V or annexin V) for 5 min before addition of dye–annexin V. Dye–annexin V conjugates (0.1 μg of each dye–annexin V in 200 μL binding buffer) were added for 10 min at room temperature. Relative binding to cells was determined as the median fluorescence from FACS analysis. Values were normalized to the median fluorescence with no added annexin V. Fluorescence microscopy was performed on an Axiovert Zeiss microscope (Carl Zeiss MicroImaging).

Tryptic digestion: $Cy5.5_{1.1}$–annexin V was reacted with iodoacetamide, digested with trypsin, and analyzed by LC/MS on a Finnegan LCQ Deca instrument at the Tufts Peptide Core Facility. Predicted weights ("MW pred," Table 1) of the peptides obtained were calculated from PeptideMass software on the ExPASy web site. Observed molecular weights ("MW obs," Table 1) were obtained from an analysis of all MS spectra throughout the HPLC chromatogram by using Sequest software.[21]

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