

## New Insights into the tPA-Annexin A2 Interaction

IS ANNEXIN A2 CYS<sup>8</sup> THE SOLE REQUIREMENT FOR THIS ASSOCIATION?\*

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Oriol Roda<sup>‡§¶</sup>, M. Luz Valero<sup>‡</sup>, Sandra Peiró<sup>‡</sup>, David Andreu<sup>‡¶</sup>, Francisco X. Real<sup>‡§¶</sup>,  
and Pilar Navarro<sup>§\*\*</sup>

From the <sup>‡</sup>Departament de Ciències Experimentals i de la Salut, Facultat de Ciències de la Salut i de la Vida, Universitat Pompeu Fabra, 08003-Barcelona, Spain and the <sup>§</sup>Unitat de Biologia Cel·lular i Molecular, Institut Municipal d'Investigació Mèdica, 08003-Barcelona, Spain

Annexin A2 has been described as an important receptor for tissue-type plasminogen activator in endothelium and other cell types. Interaction between tissue-type plasminogen activator and its cellular receptor is critical for many of the functions of this protease. The annexin A2 motif that mediates tissue plasminogen activator interaction has been assigned to the hexapeptide LCKLSL in the amino-terminal domain of the protein, and it has been proposed that Cys<sup>8</sup> of this sequence is essential for tPA binding. In an attempt to identify other amino acids critical for tPA-annexin A2 interaction, we have analyzed a set of peptides containing several modifications of the original hexapeptide, including glycine scans, alanine scans, D-amino acid scans, conservative mutations, cysteine blocking, and enantiomer and retroenantiomer sequences. Using a non-radioactive competitive binding assay, we have found that all cysteine-containing peptides, independently of their sequence, compete the interaction between tPA and annexin A2. Cysteine-containing peptides also inhibit tPA binding to the surface of cultured human umbilical vein endothelial cells (HUVEC). Mass spectrometry demonstrates that the peptides bind through a disulfide bond to a cysteine residue of annexin A2, the same mechanism that has been suggested for the inhibition mediated by homocysteine. These data call for a revision of the role of the LCKLSL sequence as the sole annexin A2 structural region required to bind tPA and indicate that further studies are necessary to better define the annexin A2-tPA interaction.

Tissue-type plasminogen activator (tPA)<sup>1</sup> is a serine protease that converts the zymogen plasminogen to the active enzyme

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<sup>¶</sup> Both authors contributed equally to this work.

\*\* To whom correspondence should be addressed: Unitat de Biologia Cel·lular i Molecular, Institut Municipal d'Investigació Mèdica, Dr. Aiguader, 80, 08003-Barcelona, Spain. Tel.: 34-93-2211009; Fax: 34-93-2213237; E-mail: pnavarro@imim.es.

<sup>1</sup> The abbreviations used are: tPA, tissue-type plasminogen activator; b<sup>tn</sup>tPA, biotinylated tPA; AnxA2, annexin A2; rAnxA2, recombinant annexin A2; hC, homocysteine; p11, p11 light chain of annexin A2 tetramer or S100A10; HUVEC, human umbilical vein endothelial cells; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight.

plasmin, which in turn degrades the fibrin network of thrombi and blood clots (1, 2). In addition to its important role in thrombolysis, plasmin participates in the extravascular breakdown of matrix and basement membrane in events such as cell migration, tissue remodeling, and invasive growth (3–5). tPA is mainly synthesized in vascular endothelial cells and secreted into the circulating blood as a 527-residue single-chain glycoprotein that can be further converted into the two-chain form upon specific cleavage at the Arg<sup>275</sup>-Ile<sup>276</sup> peptide bond (6).

Characterization of specific receptors for components of the fibrinolytic system has been a crucial point of interest in this area. In addition to regulating the dynamics of clot lysis, these receptors may contribute to numerous cellular functions that are dependent upon cell-surface proteolytic activity (7). Endothelial cell receptors for tPA and plasminogen are particularly relevant because of the proximity of these cells to vascular injury and fibrin deposition sites.

Annexin A2 (also termed annexin II, p36, calpactin 1, or lipocortin II) has been identified as a receptor for tPA and plasminogen (8, 9) in the surface of endothelial cells. The annexins (for a review see Refs. 10 and 11) are a family of proteins that bind to acidic phospholipids in the presence of Ca<sup>2+</sup>. All members of the annexin family contain four or more units of a conserved structural element of ~70 amino acids, designated the annexin repeat, and a highly variable amino-terminal domain believed to determine individual annexin functions. A variety of biological functions have been described for the annexins, including regulation of membrane traffic (12–14), transmembrane ion channel (15, 16), inhibition of blood coagulation (17–20), signal transduction in mitogenesis or differentiation (21–24), and regulation of cell-matrix or cell-cell interactions (25–29).

Extracellular AnxA2 has also been described as a membrane-bound receptor for a number of different molecules, although its interaction with the plasminogen system elements on the endothelial cell surface is the best characterized. Simultaneous binding of tPA and plasminogen to AnxA2 at the endothelial cell surface results in a 60-fold increase in catalytic efficiency of plasmin generation (9, 30). Previous reports (31) have mapped the tPA binding site of AnxA2 to the hexapeptide LCKLSL, corresponding to residues 7–12 of its amino-terminal domain. These results were obtained using a competitive solid phase radioligand binding assay with synthetic peptides corresponding to the AnxA2 amino-terminal domain sequence as competitors of either purified or recombinant AnxA2. Results were further confirmed *in vivo* using primary cultures of human umbilical vein endothelial cells (HUVEC). The LCKLSL sequence contains a free thiol group (Cys<sup>8</sup>) (32) that is crucial for tPA interaction. Evidence for this conclusion included i) mutation of AnxA2 Cys<sup>8</sup> to Gly resulted in loss of tPA binding,

whereas mutations in the other Cys residues of the protein (C133G, C262G, and C335G) did not (31), and ii) homocysteine (hC), an amino acid with prothrombotic properties, competed the binding of tPA to AnxA2 by forming a disulfide bond with Cys<sup>8</sup> (31).

AnxA2 is expressed not only in endothelial cells but also in other cell types (33, 34), including tumor cells (35). Interestingly, pancreas cancer cells overexpress AnxA2 (36, 37). Previous results from our group have demonstrated that tPA is also overexpressed in human pancreas tumors, and its inhibition using neutralizing antibodies or chemicals results in decreased invasiveness and tumorigenicity (37, 38). Therefore, it is important to identify tPA receptors in pancreatic cells and their involvement in tumorigenesis. The fact that AnxA2 has been shown to be overexpressed in pancreatic tumors supports the hypothesis that it could act as a tPA receptor. Recent work<sup>2</sup> indicates that AnxA2 may mediate, at least in part, the effects of tPA on pancreas cancer cells and therefore supports the notion that this protein could be used as a potential therapeutic target. To further probe this hypothesis, we evaluated a panel of synthetic peptides as competitors of the tPA-AnxA2 interaction. On the basis of the original LCKLSL motif, we generated a small library of peptides with alterations in sequence, chain length, configuration/conformation, and/or availability of the Cys-free thiol group. Our results demonstrate that all Cys-containing peptides, regardless of their sequence, are able to compete the tPA-AnxA2 interaction. MALDI-TOF mass spectrometry was used to determine the nature of the interaction between recombinant AnxA2 and Cys (LCKLSL) or homocysteine (L(hC)KLSL)-containing peptides and a Cys-lacking peptide of similar sequence (LAKLSL). In the first two instances, an increase in molecular weight compatible with a Cys and HC residue, respectively, strongly argued for the formation of the disulfide bond, whereas incubation of recombinant AnxA2 (rAnxA2) with the peptide lacking a thiol group had no effect. These results suggest that the LCKLSL hexapeptide, which has been assumed to be a key feature in tPA-AnxA2 binding, plays a distinctive, though probably not exclusive, role in such interaction, to the extent that blocking of the Cys residue will preclude the association of both proteins. Further research thus appears to be necessary to identify additional domains of AnxA2 involved in binding to tPA.

#### EXPERIMENTAL PROCEDURES

**Materials**—All reagents were purchased from Sigma unless otherwise indicated. rAnxA2 was prepared from BL21 *Escherichia coli* transformed with the pET21b(+) vector containing the human AnxA2 cDNA, kindly provided by Dr. K. A. Hajjar (Cornell University Medical College, New York, NY), as previously described (40). Purification was performed using a nickel nitrilotriacetic acid-agarose column after elution using a pH gradient from pH 8 to 4.8. Two elution peaks were collected, one at pH 5.2, corresponding to the monomeric form of the protein, and another one at pH 4.8, corresponding to the dimer. Both peaks were pooled and stored at  $-80^{\circ}\text{C}$ .

**Peptides**—Hexapeptides designed on the basis of the LCKLSL sequence (Table I) were synthesized on *p*-methylbenzhydrylamine resin as C-terminal carboxamides by solid phase methods using Boc chemistry. After HF cleavage, the peptides were purified to homogeneity ( $\geq 90\%$  by analytical HPLC) by preparative reverse phase HPLC (41). Peptides were satisfactorily characterized by amino acid analysis and by MALDI-TOF mass spectrometry (Voyager DE-STR, Applied Biosystems, Foster City, CA), which was also used to confirm their oxidative state (free thiol).

**Cell Culture**—Human umbilical vein endothelial cells (HUVEC, passage 2–6) were cultured in M199 medium with 10% fetal calf serum supplemented with heparin and endothelial cell growth factors as previously described (42).

**tPA Biotinylation**—Recombinant tPA (actilyse, Roche Molecular Biochemicals) (2 mg) was biotinylated using a 20-fold molar excess of Sulfo-NHS-LC-biotin (Pierce, Rockford, IL) for 2 h at  $25^{\circ}\text{C}$ , according to the manufacturer's instructions. Excess unreacted biotin was removed by gel filtration using Sephadex G25. The integrity of biotinylated tPA was examined by SDS-PAGE (43), and protein concentration was determined by the Bradford method (44) using the Bio-Rad protein assay (Bio-Rad).

**Non-radioactive Binding Assays**—Binding experiments were performed essentially as previously described (31, 40) except that biotinylated tPA (<sup>btnt</sup>tPA) was used instead of <sup>125</sup>I-tPA.

**rAnxA2 Binding Assays**—Ninety-six well Nunc Maxisorp plates (Nunc, Naperville, IL) were incubated with rAnxA2 (50  $\mu\text{l}$ /well at 10  $\mu\text{g}/\text{ml}$ ) overnight at  $4^{\circ}\text{C}$ . After three washes, plates were equilibrated (2 h,  $37^{\circ}\text{C}$ ) with incubation buffer (11 mM Hepes, 137 mM NaCl, 4 mM KCl, 3 mM CaCl<sub>2</sub>, 2 mM MgCl<sub>2</sub>, 1 mM glucose, 0.5% bovine serum albumin, pH 7.2) and <sup>btnt</sup>tPA (100 nM, 2 h,  $37^{\circ}\text{C}$ ) was then added in the presence or in the absence of peptides. Alternatively, peptides were directly added to rAnxA2, either directly or after washing, and <sup>btnt</sup>tPA was subsequently added. Plates were washed 3 times with 0.02% Tween 20, 0.5% bovine serum albumin in phosphate-buffered saline and then incubated for 1 h at  $37^{\circ}\text{C}$  with alkaline phosphatase-coupled streptavidin (Zymed Laboratories Inc., San Francisco, CA). Enzymatic activity was measured using 4-methylumbelliferyl phosphate (1 mg/ml in triethanolamine buffer, pH 9.5) for 20 min at room temperature. Quantification was performed using a Cytofluor 235 instrument (Millipore, Bedford, MA).

**HUVEC Binding Assays**—Confluent HUVEC, cultured in 96-well Nunclon plates (Nunc, Naperville, IL), were equilibrated for 1 h at  $37^{\circ}\text{C}$  with incubation buffer (same as above). 100 nM <sup>btnt</sup>tPA was then added for 1 h at  $37^{\circ}\text{C}$  in the absence or presence of selected peptides (10–1000  $\mu\text{M}$ ). After 3 washes with incubation buffer, cells were fixed with methanol (5 min at  $-20^{\circ}\text{C}$ ), washed 3 times with 0.02% Tween 20 in phosphate-buffered saline, and incubated with alkaline phosphatase-coupled streptavidin (1 h,  $37^{\circ}\text{C}$ ). Reactions were developed and quantified as described above. To determine specific binding, a 50-fold excess of unlabeled tPA was added during the incubation with <sup>btnt</sup>tPA.

**Mass Spectrometry**—rAnxA2 (450  $\mu\text{M}$  in phosphate-buffered saline) was incubated with a 5-mM solution of peptide (LCKLSL, L(hC)KLSL or LAKLSL) and with 5 mM free homocysteine (hC) for 3 h at  $37^{\circ}\text{C}$ . The pH was adjusted to 7.4 with NH<sub>4</sub>CO<sub>3</sub> before the incubation. The reaction was stopped with 1  $\mu\text{l}$  of formic acid and diluted 1:5 with 50% methanol, 0.1% trifluoroacetic acid. One microliter of the resulting solution was mixed with sinapinic acid (1:1) and analyzed by MALDI-TOF mass spectrometry (Voyager DE-STR, Applied Biosystem). The direct binding of LCKLSL, L(hC)KLSL, and LAKLSL peptides to rtPA was analyzed under the same experimental conditions.

#### RESULTS

**Amino Acid Modifications of the LCKLSL AnxA2 Sequence**—Previous work led to the identification of the hexapeptide LCKLSL (residues 7–12) from the AnxA2 amino-terminal domain as the minimum sequence required for interaction between AnxA2 and tPA (31). Replacement of the Cys<sup>8</sup> of this sequence by Gly abolished the interaction with tPA, indicating that this amino acid is critical for such interaction. To determine whether additional amino acids of this hexapeptide are required for the tPA-AnxA2 interaction and to identify peptides with improved blocking properties, we designed a small hexapeptide library with the modifications summarized on Table I. These modifications include glycine scans (peptides 2–7), alanine scans (peptides 8–13), D-amino acid scans (peptides 14–19), conservative replacements (Leu/Ile, peptides 20–22; Leu/Val, peptides 23–25; Lys/Arg, peptide 26; Ser/Thr, peptide 27), Cys protection (peptide 28), and enantiomer and retroenantiomer sequences (peptides 29 and 30). The competitive properties of the peptides were then evaluated in the tPA-AnxA2 interaction assays described below.

**Receptor Binding Properties of Biotinylated tPA**—We employed a non-radioactive modification of the competitive binding experiments already described (31) using tPA labeled with biotin (see “Experimental Procedures” for details). Fig. 1 shows

<sup>2</sup> S. Peiró, S. Aguilar, J. M. Corominas, F. X. Real, and P. Navarro, unpublished work.

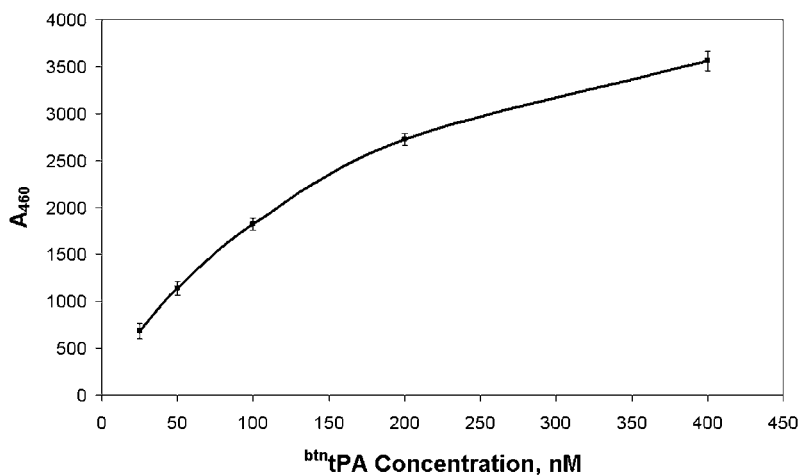
TABLE I  
Synthetic peptides used to compete the binding of tPA to AnxA2

Synthetic peptides containing the indicated modifications were obtained on the basis of the canonical hexapeptide 7–12 amino-terminal motif of annexin A2. The ability of all peptides to compete the interaction between biotinylated tPA and AnxA2 was analyzed as described under “Experimental Procedures.”

Modification	Sequence	Peptide number	tPA binding inhibition <sup>a</sup>
Canonical Gly scan	LCKLSL	1	+++
	GCKLSL	2	++
	LGKLSL	3	–
	LCGLSL	4	+++
	LCKGSL	5	++
	LCKLGL	6	+++
	LCKLSG	7	++
Ala scan	ACKLSL	8	++
	LAKLSL	9	–
	LCALSL	10	++
	LCKASL	11	+++
	LCKLAL	12	+++
	LCKLSA	13	+++
	ICKLSL	14	+++
D-Amino acid scan	LcKLSL	15	++
	LcKLSL	16	+++
	LCKISL	17	++
	LCKLsL	18	+++
	LCKLSI	19	+++
	ICKLSL	20	++
	LCKISL	21	+++
Conservative replacements (underlined)	LCKLSI	22	+++
	<u>V</u> CKLSL	23	++
	LCKVSL	24	++
	LCKLSV	25	++
	LCRLSL	26	++
	LCKLTL	27	+++
	LC [Acm] KLSL	28	–
Cys protection	lcklsl	29	+++
	lslkcl	30	+++
Enantiomer			
Retroenantiomer			
Unrelated	SFQTTTTYPSPHPQTTLPC	31	+++
Random	KLLCLS	32	+++
Tetrapeptide	LCKL	33	+++
Cys/hC replacement	L (hC) KLSL	34	+++

<sup>a</sup> tPA binding inhibition was considered as: +++, >60%, ++, 40–60%, and –, <10% at 100 nM biotinylated tPA and 100 μM peptide concentration.

FIG. 1. Binding of <sup>btnt</sup>tPA to recombinant AnxA2. Wells of a 96-well plate were coated with rAnxA2 as described under “Experimental Procedures” and incubated with increasing concentrations of <sup>btnt</sup>tPA. Results of one representative experiment of two independent assays performed are shown. Error bars indicate S.E. ( $n = 3$ ).



that soluble <sup>btnt</sup>tPA interacts in a dose-dependent fashion with immobilized rAnxA2, showing half-maximal binding at a 200 nM concentration. This binding profile is in the same range as that already described for <sup>125</sup>I-tPA (8), indicating the comparability of both assays. Moreover, an excess of unlabeled tPA completely inhibited the binding of <sup>btnt</sup>tPA to AnxA2 (not shown).

In addition, cell binding studies were performed by assaying the binding of <sup>btnt</sup>tPA to HUVEC cells because AnxA2 is the main receptor for tPA in endothelial cells. As shown in Fig. 2, <sup>btnt</sup>tPA binds to the surface of HUVEC in a selective and dose-

dependent manner, indicating that the labeled protein also recognizes native AnxA2 on the cell membrane.

*Competitive tPA-AnxA2 Binding Properties of LCKLSL and Modified Hexapeptides in Vitro and in Vivo*—The peptides described in Table I were tested for their ability to compete with the interaction between <sup>btnt</sup>tPA and rAnxA2. Fig. 3 shows the results obtained with several representative peptides. As expected, peptides lacking Cys<sup>8</sup> (Fig. 3, peptide 9, LAKLSL) did not compete for tPA binding; the Ala substitution is shown, but similar results were obtained with the Gly substitution and when the Cys was protected, confirming the crucial role of this



FIG. 2. **Binding of  $^{btnt}$ tPA to HUVEC.** Confluent HUVEC cultured in 96-well plates were incubated with increasing concentrations of  $^{btnt}$ tPA. Results of one representative experiment of two independent assays performed are shown. Error bars indicate S.E. ( $n = 3$ ).

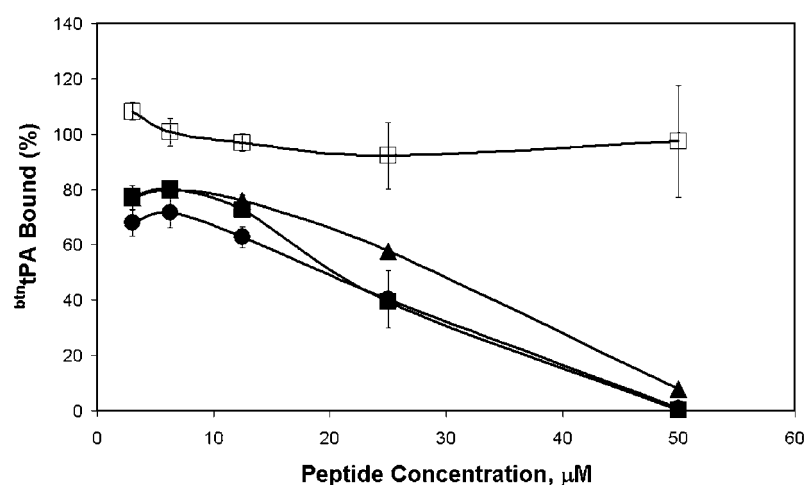
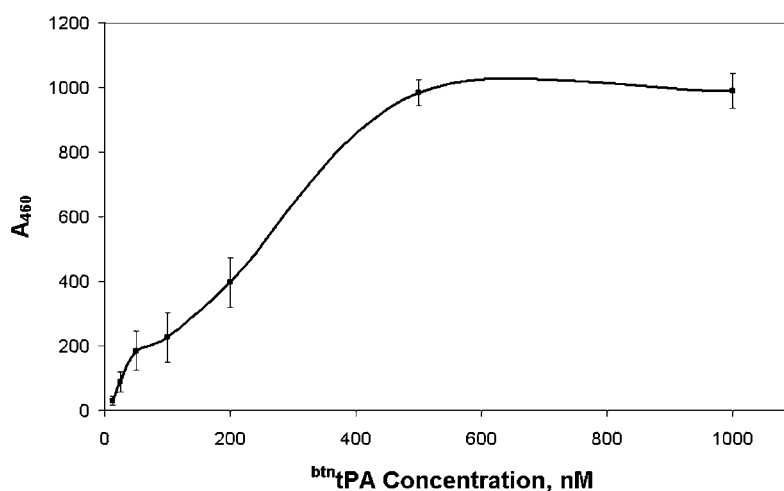


FIG. 3. **Effect of synthetic LCKLSL-derived hexapeptides on  $^{btnt}$ tPA binding to rAnxA2.** Wells of a 96-well plate were coated with rAnxA2 as described under "Experimental Procedures." Peptides, at the indicated concentrations, and  $^{btnt}$ tPA (100 nM) were simultaneously added to rAnxA2 and incubated at 37 °C for 2 h. Peptide 1 (LCKLSL), ■, Peptide 15 (LAKLSL), ▲, Peptide 29 (lcklsl), □, Peptide 30 (lslkcl), ●. Results of one representative experiment of two independent assays performed are shown. Error bars indicate S.E. ( $n = 3$ ).

amino acid in the interaction. Surprisingly, the enantiomer (Fig. 3, peptide 29, lcklsl) and the retroenantiomer (Fig. 3, peptide 30, lslkcl) competed the interaction in a manner similar to that of the canonical hexapeptide (Fig. 3, peptide 1, LCKLSL), indicating that configurational/conformational characteristics are not determinant for the binding. Moreover, all other amino acid modifications resulting from the Ala and Gly scans showed the same competition activity as the LCKLSL sequence (Table I).

We next assayed the inhibitory effect of the modified hexapeptides on tPA binding to the surface of endothelial cells. Fig. 4 shows that the canonical hexapeptide (peptide 1, LCKLSL) and its enantiomer (peptide 29, lcklsl) cause a 40–60% reduction of the binding of  $^{btnt}$ tPA to HUVEC. Peptides with mutations in Cys had no effect (peptide 9, LAKLSL, is shown in Fig. 4).

**All Cysteine-containing Peptides Compete the Binding of tPA-AnxA2**—The results described above strongly suggested that the presence of a Cys residue in the sequence was sufficient to compete the tPA-AnxA2 interaction. To test this hypothesis, a completely unrelated peptide containing one Cys residue (peptide 31, SFQTTTTYPTSPHPQTTLPC), a randomized version of the LCKLSL sequence (peptide 32, KLLCLS), and a four-amino acid peptide fragment of the canonical hexapeptide (peptide 33, LCKL) were next assayed. As shown in Fig. 5, all these peptides display the same competitive activity as LCKLSL, indicating that the presence of a Cys residue in the competing peptide is sufficient to disrupt the interaction.

To determine whether binding of peptides to tPA, rather

than to AnxA2, was required for the inhibition, peptides were directly added to rAnxA2, plates were washed, and  $^{btnt}$ tPA was then added. The results were similar to those described above, strongly suggesting the direct interaction of AnxA2 with the peptides (data not shown).

**Homocysteine Inhibits the Binding of Biotinylated tPA to AnxA2**—The specific interaction of tPA with endothelial cells is inhibited by the thiol-containing amino acid hC, a fact that has been postulated as an explanation for the thrombotic events related to homocysteinemia (45–47). The molecular mechanism by which hC impairs tPA-endothelium interaction has been proposed to be based on the direct blockade of the tPA binding domain of AnxA2 through formation of a disulfide link with Cys<sup>8</sup> (31). We have compared the effects of hC and the peptides used in this study on the binding of tPA to AnxA2 under the experimental conditions described above. Fig. 6 shows that hC competed less efficiently than the peptides. In contrast, when an analogue of the canonical hexapeptide containing hC instead of Cys (peptide 34, L(hC)KLSL) was assayed, it competed as efficiently as the consensus LCKLSL peptide. The lower effect of free *versus* peptide-incorporated hC may be explained by differences in stability of the reduced state.

**Cysteine-containing Peptides Form a Disulfide Linkage with rAnxA2**—As mentioned above, the mechanism proposed to explain the blocking effect of hC on the tPA-AnxA2 interaction is direct binding between hC and Cys<sup>8</sup> through a disulfide bond (31). Our previous results suggest that any Cys-containing sequence can compete the binding between tPA and rAnxA2 in a similar fashion. To account for this fact

FIG. 4. **Effect of synthetic LCKLSL-derived hexapeptides on <sup>btnt</sup>tPA binding to HUVEC.** HUVEC were cultured in 96-well plates and grown to confluence as described under "Experimental Procedures." <sup>btnt</sup>tPA (100 nM) was added in presence of peptide 1 (LCKLSL), ■, peptide 15 (LAKLSL), □, and peptide 29 (lcklsl), ▲. Results of one representative experiment of three performed are shown.

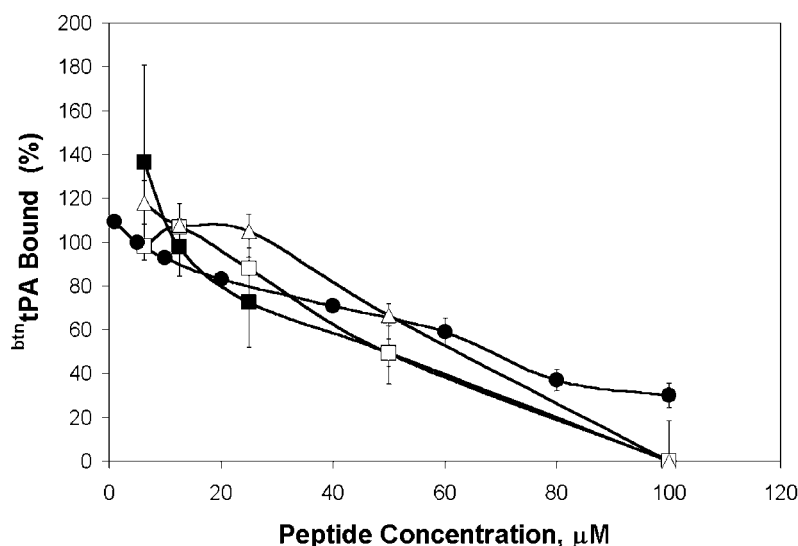
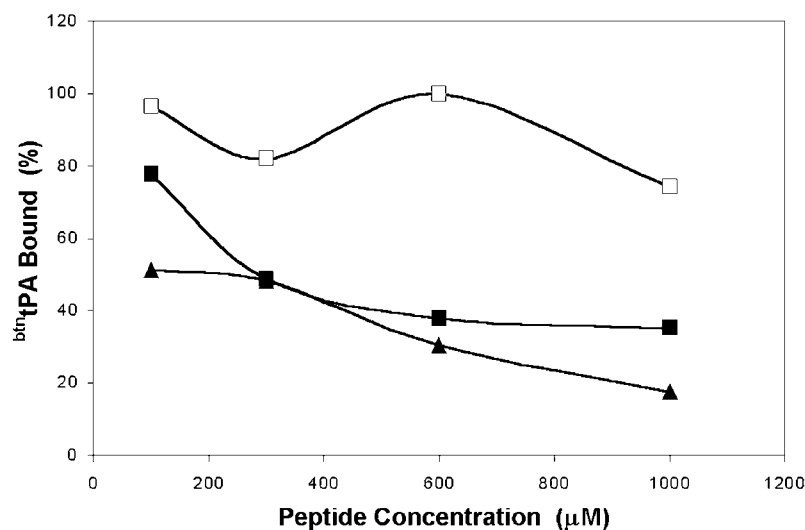
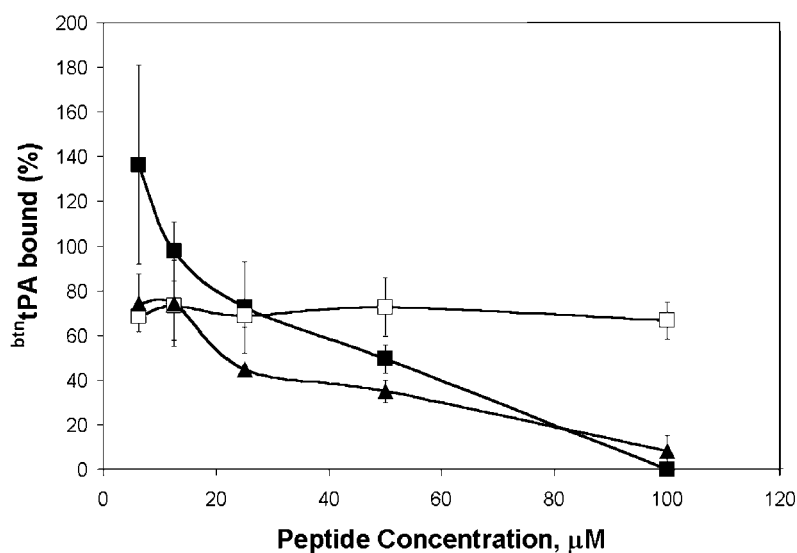


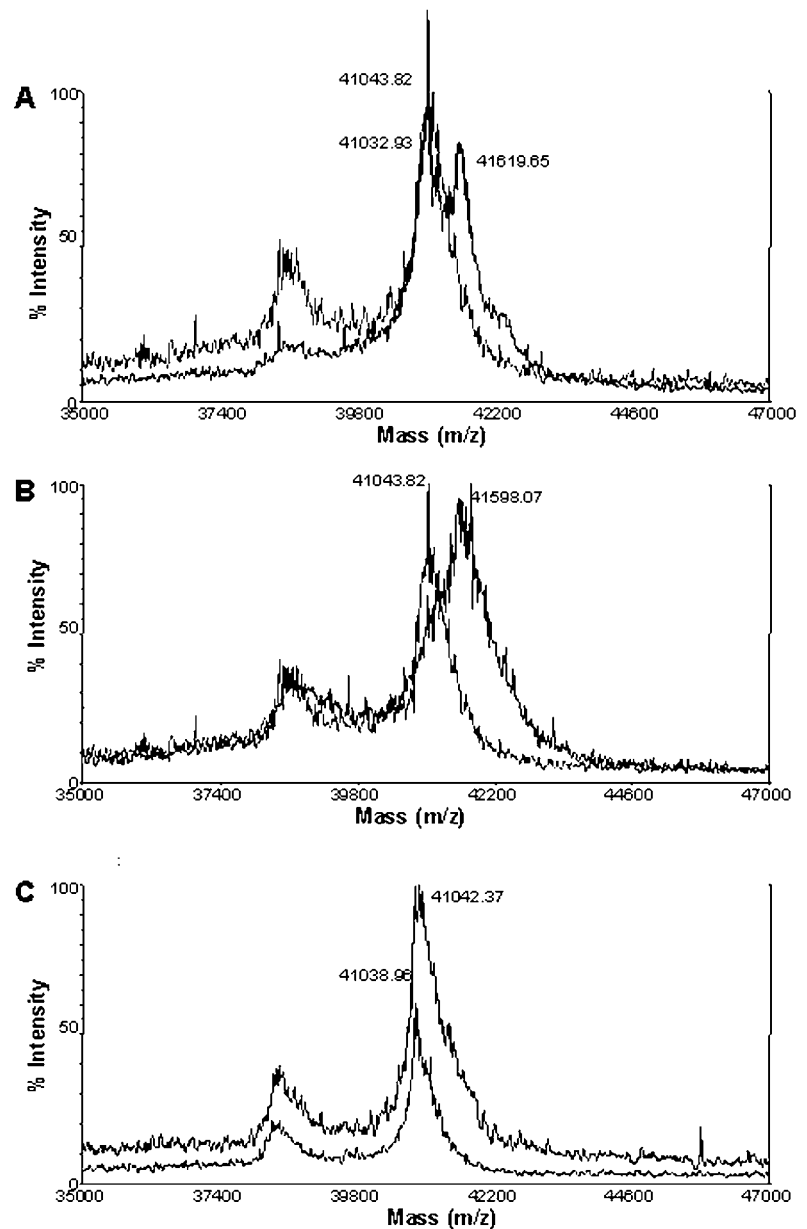
FIG. 5. **Effect of Cys-containing synthetic peptides on <sup>btnt</sup>tPA binding to rAnxA2.** Wells of a 96-well plate were coated with rAnxA2 as described under "Experimental Procedures." Peptides, at the indicated concentrations, and <sup>btnt</sup>tPA (100 nM) were added to rAnxA2 and incubated at 37 °C for 2 h. Peptide 1 (LCKLSL), ■, Peptide 32 (KLLCLS), □, Peptide 33 (LCKL), ▲, Peptide 31 (SF-GTTTTTYPTPSHPQTTLPC), ●. Results of one representative experiment of three independent assays performed are shown. Error bars indicate S.E. ( $n = 3$ ).

FIG. 6. **Effect of homocysteine and hC-containing synthetic peptide on <sup>btnt</sup>tPA binding to rAnxA2.** Wells of a 96-well plate were coated with rAnxA2 as described under "Experimental Procedures." Peptides, at the indicated concentrations, and <sup>btnt</sup>tPA (100 nM) were added to rAnxA2 and incubated at 37 °C for 2 h. Peptide 1 (LCKLSL), ■, Homocysteine, □, Peptide 34 [L(hC)KLSL], ▲. Results of one representative experiment of two performed are shown. Error bars indicate S.E. ( $n = 3$ ).



we have considered two possible explanations: i) a low affinity interaction (disrupted by high concentrations of thiol-containing peptides) exists between tPA and AnxA2, which implicates Cys<sup>8</sup> of AnxA2, or ii) by forming a disulfide bond

with Cys<sup>8</sup> of AnxA2 (as does homocysteine), Cys-containing peptides render this amino acid unavailable for further interaction, such as tPA binding. To test these hypotheses, we have studied the binding of Cys-containing peptides to



**FIG. 7. Mass spectrometry analysis of untreated and peptide-treated rAnxA2.** rAnxA2 was dissolved in phosphate-buffered saline, incubated with different peptides for 3 h at 37 °C, and analyzed by MALDI-TOF mass spectrometry as described under "Experimental Procedures." Analyses of untreated rAnxA2 (panels A, B, and C, left graph) as well as rAnxA2 treated with peptide 1 (LCKLSL) (panel A, right graph), peptide 34 (L(hC)KLSL) (panel B, right graph), or peptide 15 (LAKLSL) (panel C, right graph).

rAnxA2 by mass spectrometry. Fig. 7 shows the MALDI-TOF spectra of AnxA2 incubated, or not, with the hexapeptides LCKLSL (panel A) and L(hC)KLSL (panel B). Both peptides bound to rAnxA2, as reflected by the increase of molecular mass from 41,043 Da (free rAnxA2) to 41,619 Da (rAnxA2 incubated with LCKLSL) or 41,598 Da (rAnxA2 incubated with L(hC)KLSL). These increments in 576 and 555 Da, respectively, fit well with the molecular mass of the hexapeptides and suggest the formation of a complex with a 1:1 stoichiometry. In contrast, incubation of rAnxA2 with the Cys-lacking hexapeptide LAKLSL had no effect on the mass spectrum (Fig. 7, panel C). These results indicate that peptide competition is mediated by direct disulfide bond formation with rAnxA2. This interaction is likely to be mediated by Cys<sup>8</sup>, although the participation of other Cys residues cannot be completely ruled out.

To exclude the possibility that the peptides bound promiscuously to any Cys-containing protein, and more specifically to tPA, LCKLSL, L(hC)KLSL, and LAKLSL peptides were incubated with tPA, and the reaction products were analyzed by MALDI-TOF. The spectra of tPA were not significantly

changed upon incubation with any of the peptides analyzed (data not shown).

#### DISCUSSION

The dramatic increase in tPA catalytic activity resulting from its binding to cellular membranes provides an interesting target for the modulation of its biological functions related to the proteolytic degradation of fibrin as well as other substrates. We have recently shown that tPA is overexpressed in pancreas cancer cells and the blockade of tPA is associated with reduced *in vitro* invasiveness and tumorigenicity (37, 38). Therefore, the tPA system constitutes an attractive target for the development of novel therapies. Because cell surface receptors allow focalization of the proteolytic activity at the cell membrane and may also participate in signal transduction, their identification is of major importance.

AnxA2 has been shown to be a major tPA receptor in endothelial cells, among others, and preliminary data indicate that this protein is also overexpressed in pancreas cancers (36, 37).<sup>2</sup> On the basis of these findings, it can be postulated that AnxA2 might play a role in stimulating tPA activity and plasmin

generation at the membrane of cancer cells, potentially leading to increased tumorigenic properties. Nevertheless, our data do not exclude the possibility that some of the effects of tPA and its receptor(s) take place independently of its proteolytic activity and/or that of plasmin, as is the case of some effects mediated by the receptors for urokinase-type plasminogen activator (48). The blockade of the interaction between tPA and AnxA2 might provide clues about the precise role of this molecule as a tPA cell surface receptor in cancer cells.

Hajjar *et al.* (31) have recently reported on the critical role of a Cys residue in the LCKLSL (residues 7–12) sequence at the N terminus of AnxA2 for tPA and hC binding and have proposed that the interaction between the latter and AnxA2 leads to a reduction of tPA binding, thus providing an explanation for the prothrombotic effects of hC (45–47). This conclusion was based on the fact that: 1) an AnxA2 33-kDa chymotryptic peptide lacking the N-terminal peptide did not compete tPA-AnxA2 binding, 2) the hexapeptide abolished the binding of  $^{125}\text{I}$ -tPA to immobilized purified AnxA2 and to HUVEC cells, 3) synthetic peptides lacking Cys<sup>8</sup> did not inhibit binding, and 4) a systematic mutation of all Cys residues of AnxA2 showed that only Cys<sup>8</sup> was required for the interaction. Therefore, we initiated a search for variant peptides that were able to better compete AnxA2-tPA binding and had more suitable pharmacological properties. These peptides were tested using the experimental procedures previously described (31) except that tPA was labeled with biotin instead of  $^{125}\text{I}$ . Our findings confirm the requirement of Cys in the LCKLSL sequence but also suggest that other regions of the molecule must be necessary in order to confer specificity to the interaction.

We found that all peptides containing a Cys residue, regardless of the rest of their sequence and of the position in which the Cys was located, were able to compete the interaction between tPA and AnxA2 because of their capacity to bind AnxA2. The fact that the consensus peptide, as well as its enantiomer and retroenantiomer versions, showed similar properties in these assays and the small size of the peptides used argue in favor of mechanisms independent of a strong secondary structure. Because hC has been shown to block AnxA2-tPA binding through the formation of a disulfide bond with the thiol group of Cys<sup>8</sup>, we considered the possibility that the effects of synthetic peptides used in the assays performed here and in the work of Hajjar *et al.* might act through similar mechanisms. Our results indicate that the peptides compete with AnxA2-tPA binding more efficiently than hC and that peptide LCKLSL and its enantiomer bind covalently to AnxA2 and induce an increase in molecular mass, as determined by mass spectrometry, whereas a peptide with a Cys-Ala substitution does not bind. Although we cannot exclude the covalent binding of the peptides to other Cys residues, all available evidence points to an interaction with Cys<sup>8</sup>, because it has been shown that it is the only residue to which hC binds (31). Similar analyses using mass spectrometry showed that the same peptides do not bind tPA.

AnxA2 has been described (49) to exist as at least three different forms, monomer, heterodimer (composed of one molecule of AnxA2 and one molecule of 3-phosphoglycerate kinase), and heterotetramer (two AnxA2 subunits and two 11-kDa regulatory subunits called p11 or S100A10). These different forms seem to be present in distinct subcellular compartments; AnxA2 monomer is mainly cytosolic (49–51), whereas the heterodimer has been described in the nucleus (52–54) and the heterotetramer is associated with the plasma membrane (55). The AnxA2 heterotetramer is the most abundant form of the protein, representing 90–95% of the total AnxA2 in endothelial, epithelial, and Madin-Darby canine kidney cells (34, 56). The p11 light chain regulates many of the

activities of AnxA2 and confers to the heterotetramer biochemical properties distinct from those of the monomer (49, 57, 58). In particular, the tetramer is an extremely potent activator of plasminogen, stimulating the rate of activation of (Glu) plasminogen about 341-fold, compared with an approximate 6-fold stimulation by the monomer, and inducing a 90-fold increase in the catalytic efficiency of tPA for (Glu) plasminogen (59). Binding of p11 to AnxA2 also decreases the  $K_d$  ( $\text{Ca}^{2+}$ ) for the binding of AnxA2 to biological membranes. In addition, the monomer bundles F-actin to a much lesser extent than the tetramer, and actin binding is important for regulation of intracellular AnxA2-membrane-mediated functions. Overall, the data suggest that p11 acts as a modulator of properties displayed by the p36 core protein. Interestingly, the interaction domain for p11 has been located, using fluorescence spectroscopy, within the first 9 amino acids of the AnxA2 tail (STVHEILCK) (32). This sequence partially overlaps with the LCKLSL peptide, suggesting that both p11 and tPA could compete for binding to AnxA2. However, the fact that tPA can bind to the tetramer, thereby leading to enhanced plasmin generation activity, supports the notion that non-overlapping domains must also be involved in binding of AnxA2 to p11 and to tPA. Our studies do not modify the interpretation of the mechanism of interaction of AnxA2 with p11, because the latter has been shown with a fluorophore used in fluorescence spectroscopy studies to be dependent on the N-terminal acetyl group of Ser<sup>1</sup> (60, 61) and unaffected by substitution of Cys<sup>8</sup> (32). Because the AnxA2 tetramer is overexpressed at the extracellular side of the membrane in tumor cells (35) and its expression has been associated with cellular transformation and metastasis (35, 28), elucidating the precise structure of the complex remains an important task.

Regarding the binding site in the tPA molecule, Beebe *et al.* (62) have proposed that residues 7–17 of its finger domain (RDEKTQMIYQQ) are involved in binding to AnxA2. This sequence mimics partially the sequence of p11 responsible for AnxA2 binding (CRDGK, residues 61–65), suggesting again the possibility of competition between both molecules for AnxA2 binding. However, the high concentrations (mM) of tPA-derived peptide required for HUVEC binding suggest that other regions of the molecule may also play a role. For instance, tPA deletion mutants lacking the finger domain or both the finger and growth factor domains are still able, although to a much lesser extent, to bind to endothelial cells (63) and stimulate plasminogen activation (39). However, in these assays the cellular receptors involved in the interaction with tPA or tPA-derived fragments were not molecularly characterized. Altogether, the available evidence indicates that a reassessment of the domains involved in the binding of AnxA2, tPA, and p11 is necessary and that it is important to take into account that different domains may be involved in different cellular processes, given the broad range of functions ascribed to the molecules involved in this complex.

The precise mechanisms through which the overexpression of tPA and AnxA2 may contribute to tumor progression in pancreas cancer, as well as in other tumor types, are not clear and may be severalfold. To tackle more effectively such processes and to develop therapeutic strategies, a better understanding of the molecular interactions between these two proteins is necessary.

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**New Insights into the tPA-Annexin A2 Interaction: IS ANNEXIN A2 CYS8 THE SOLE REQUIREMENT FOR THIS ASSOCIATION?**

Oriol Roda, M. Luz Valero, Sandra Peiró, David Andreu, Francisco X. Real and Pilar Navarro

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