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Serum leucine-rich alpha-2-glycoprotein-1 binds cytochrome *c* and inhibits antibody detection of this apoptotic marker in enzyme-linked immunosorbent assay

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Abstract Cytochrome *c* (Cyt *c*) has been implicated as a serum marker for aberrant apoptosis and, thus, has considerable clinical potential. Using a sandwich enzyme-linked immunosorbent assay (ELISA) we found that the sensitivity of Cyt *c* detection is reduced in the presence of serum. The inhibitory factor responsible was purified from both fetal bovine serum and human serum employing standard chromatography procedures followed by affinity chromatography on Affi-Gel 10-bound Cyt *c*. In SDS-PAGE, bands at 44 kD and 50 kD were observed for the bovine and human proteins, respectively. Mass spectrometry analysis identified the serum inhibitory factor as leucine-rich alpha-2-glycoprotein-1 (LRα2GP1). This identification may lead to a modified ELISA to quantify total Cyt *c* in patients' sera. LRα2GP1 is the first extracellular ligand for Cyt *c* that has been identified. A physiological function associated with binding is suggested.

Keywords Cytochrome *c* · Apoptotic marker · ELISA · Leucine-rich alpha-2-glycoprotein-1 · Leucine-rich repeats

Abbreviations BLAST: basic local alignment search tool; BSA: bovine serum albumin; Cyt *c*: cytochrome *c*; DEAE: diethylaminoethyl; ELISA: enzyme-linked immunosorbent assay; HRP: horseradish peroxidase; mAbs, monoclonal antibodies; LRα2GP1: leucine-rich alpha-2-glycoprotein-1; MALDI-TOF MS: matrix assisted laser desorption ionization time-of-flight mass spectrometry; PBS: phosphate-buffered saline; SDS-PAGE: polyacrylamide gel electrophoresis in sodium dodecylsulfate

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Introduction

In addition to its role in electron transport, the mitochondrial protein cytochrome *c* (Cyt *c*) is an important initiator/amplifier of programmed cell death or apoptosis [1] As a moonlighting protein, Cyt *c* has been implicated in a number of apoptotic functions (reviewed in ref. [2]). Following its translocation to the cytoplasm Cyt *c* binds Apaf-1 and serves as a cofactor in caspase-9 activation [3–5] Cyt *c* has also been reported to enhance calcium release by inositol trisphosphate receptors in the endoplasmic reticulum [6] and to induce acetylated histone 2A efflux from the nucleus into the cytoplasm [7]. Phospholipid-bound Cyt *c* has increased peroxidase activity and has been suggested to be involved in oxidation of cardiolipin allowing for Cyt *c* translocation from mitochondria and in oxidation of phosphatidylserine leading to externalization of the phospholipid on the plasma membrane [8].

Cyt *c* is ultimately released from apoptotic cells *in vitro* as a monomer in its native conformation [9]. This release begins to occur soon after changes in the plasma membrane that signal uptake by phagocytic cells predicting that Cyt *c* would appear outside apoptotic cells *in vivo,* particularly in situations where apoptosis is increased or when phagocytosis is defective [10]. Extracellular Cyt *c* may play a role in inflammation as it has been reported to cause arthritis when injected into mice and to induce *in vitro* the expression

of a key pro-inflammatory transcription factor, NF-κB, although at a relatively high concentration of Cyt *c* (100 μg/ml) [11].

Cyt *c* has considerable clinical potential as a serum marker for aberrant apoptosis (reviewed in ref. [2]). In several clinical trials an increase in serum Cyt *c* has been observed in a variety of patients including those with cancer [10, 12], myocardial infarcts [13, 14], apoptosis-associated liver disease [15], systemic inflammatory response syndrome [16], and influenza virus-induced encephalopathy [17]. The association of serum Cyt *c* with a number of diseases involving apoptosis indicates that it will likely not be of diagnostic value, although it may have considerable importance in prognosis as a marker for apoptotic activity in an individual.

For effective application as a clinical marker it is critical that the sensitivity of Cyt *c* detection be optimized. Sandwich ELISA (enzyme-linked immunosorbent assay) is often used to quantify antigens in a complex solution such as serum [18]. Employing monoclonal antibodies (mAbs) against the two major epitopes on Cyt *c* around the regions of residues 44/47 and residues 60/62 on the opposite surface [19–21], a sandwich ELISA can be used to detect this antigen. Several sandwich ELISA kits for Cyt *c* are commercially available and these assays have been employed to detect Cyt *c* in clinical samples [12–17]. However, we have found that serum interferes with the quantification of Cyt *c* in sandwich ELISA reducing the sensitivity of Cyt *c* detection. This may undermine the usefulness of Cyt *c* as a prognostic indicator employing currently available assays, particularly in less acute forms of apoptotic diseases where the level of circulating Cyt *c* may be only slightly elevated. Here we describe the serum interference and identify the inhibitor.

Materials and methods

Antigens, antibodies, and other reagents

Human Cyt *c* was obtained from R&D Systems, Inc. Rat and horse Cyts *c*, bovine serum albumin (BSA), horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, Lipidex 1000 (hydroxyalkoxypropyl-dextran Type VI), and all other reagents were obtained from Sigma-Aldrich unless stated otherwise. Horse and fetal bovine sera were obtained from Gibco-Invitrogen and Biotechnics Research, Inc., respectively. Human and mouse sera were obtained from living donors. mAbs employed in this study were prepared as previously described [20]. and purified by affinity chromatography using Cyt *c* coupled to CNBr-activated Sepharose 4B [22].

Sandwich ELISA

The sandwich ELISA utilized mAbs directed against two different epitopes on Cyt *c*. In this assay a mAb against one Cyt *c* epitope was attached to a well of an assay plate, followed by either the Cyt *c* antigen itself or serum/BSA mixed with Cyt *c*. A mAb against the second epitope tagged with HRP was used to detect Cyt *c* bound in the well employing the catalytic activity of HRP. In the sandwich ELISA for human Cyt *c*, mAb 2B5, specific for the region around residues 44/47, was used to capture Cyt *c* and mAb 2G8, specific for the region around residues 60/62, was used to detect bound Cyt *c*. For detection of mouse/rat Cyt *c* mAbs 1G1 and 2G8 were employed in the sandwich ELISA. The mAbs used to capture Cyt *c* were adsorbed to Nunc Immunosorb 96-well plates (Gibbco Scientific, Coon Rapids, MN) at 2.5 μg/ml in 50 μl phosphate-buffered saline (PBS). Antigen capture (approximately 8 nM) occurred in a total volume of 75 μl containing 1 mg/ml BSA (>99% pure). The mAbs used for detection were covalently coupled to HRP as has been described [18], and were used at 150–600 fold dilutions in 1 mg/ml BSA (>99% pure). All incubations were for at least 1–2 hrs and plates were washed twice in PBS containing 0.1% Triton X-100 between each incubation. The HRP reaction was carried out for 15 min. with 100 μl hydrogen peroxide (30% stock solution, 0.5 μl/ml) in citrate-phosphate buffer pH 5.0 and *o*-phenylenediamine (0.4 mg/ml), followed by 50 μl 4 N sulfuric acid to stop the reaction [18]. The absorbance at 492 nm was recorded.

Partial purification of the serum inhibitory factor

The serum inhibitory factor was first precipitated from fetal bovine serum in saturated ammonium sulfate (60–80% cut). The precipitate was resolubilized and dialysed against 10 mM phosphate pH 7.0. The dialysate was bound to diethylaminoethyl (DEAE) Sephacel and eluted at 65 mM sodium chloride in a step gradient to 200 mM. The major peak was dialyzed against 10 mM ammonium bicarbonate, lyophilized, and applied to a Sephadex *G*-100 column (50 × 1.5 cm) equilibrated in PBS, pH 7.4. The factor was partially purified from human serum following a similar protocol: 50–80% saturated ammonium sulfate cut and elution from DEAE-Sephacel between 50 and 70 mM sodium chloride in 10 mM sodium phosphate, pH 7.0. The final step involved affinity chromatography on horse Cyt *c*-Affi-Gel 10 (see below)

Amino acid sequencing

The amino terminal amino acid sequence of the major protein in the inhibitory fraction of fetal bovine serum was

determined by Edman degradation employing a Hewlett Packard 241 Protein Sequencer.

Affinity chromatography

Since the inhibitory factor in fetal bovine serum co-purified with BSA in preliminary experiments, we used a commercial preparation of impure BSA (96–99% pure) to isolate the factor by adsorbing it to horse Cyt *c* coupled to Affi-Gel 10 (Bio-Rad Laboratories). Lysozyme coupled to Affi-Gel 10 was employed as a control. The adsorbents were each coupled to 2 ml of gel at a density of 5 mg/ml. The BSA preparations (>99% pure and 96–99% pure, $\leq$40 mg/ml in PBS) were incubated on the columns for 1 h followed by washing of the columns with 20 ml PBS. Bound material was eluted in 0.5 M acetic acid, frozen at -80°C, and lyophilized. To remove lipids, BSA (40 mg, 96–99% pure) in 50 mM potassium phosphate, pH 7.4 was passed through a column containing 1.4 ml Lipidex 1000 at 37°C [23].

SDS-PAGE

SDS-PAGE was performed using 4–20% Precise Pre-cast gels and protein bands were stained using GelCode Blue (Pierce Biotechnology). Precision Plus Protein Standards were obtained from Bio-Rad.

Mass spectrometry

Gel slices containing the inhibitory factor cut from a polyacrylamide gel were in-gel digested overnight in 5% trypsin (Promega, Madison, WI). The eluate from Cyt *c*-Affi-Gel 10 was similarly digested. The digests were desalted using C18 ZipTips (Millipore, Billerica, MA). Matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MS/MS were then performed [24]. Full scans of the peptide mixtures from 500–3500 m/z and tandem mass spectral data of selected ions were collected on a QSTAR quadruple TOF mass spectrometer with an orthogonal MALDI source (Applied Biosystems, Inc., Foster City, CA) using alpha-cyano-4-hydroxycinnamic acid as the matrix. Mass spectra were averaged from approximately 50–100 laser shots collected in positive mode.

Results

Serum inhibition of Cyt *c* detection in sandwich ELISA

Sera from different species (horse, human, mouse, and fetal bovine sera) were examined for inhibition of rat and human Cyt *c* detection in the sandwich ELISA. Inhibition of Cyt *c* detection in ELISA was observed with sera from all species tested as shown in Fig. 1 in the detection of rat Cyt *c*. Slight inhibition was observed at serum concentrations below 1% and was essentially complete at 10%. Similar results were observed in the sandwich ELISA employed to detect human Cyt *c* (results not shown).

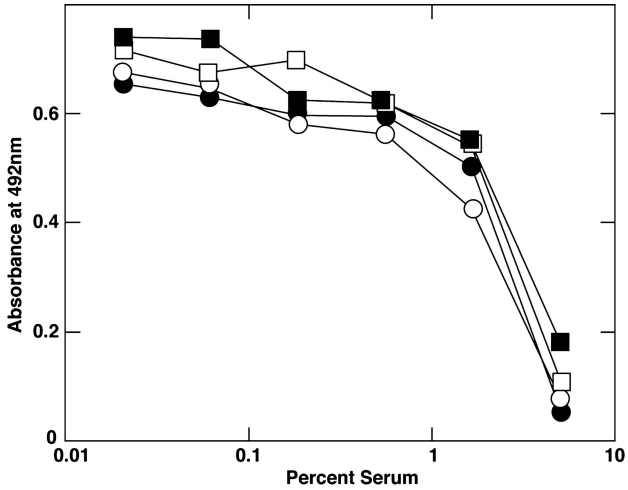

Fig. 1 The inhibitory factor for rat Cyt *c* detection in sandwich ELISA is present in human (●), mouse (○), and both normal (■) and gamma globulin-free (□) horse sera. In another experiment not shown, inhibition was also observed with fetal bovine serum. The values plotted represent the averages of triplicate readings with an average S.D. = 0.028

Since gamma globulin-free horse serum was also inhibitory in this assay, the factor responsible is not a naturally-occurring antibody reactive with Cyt *c* (see Fig. 1). It was conceivable that inhibition could have been caused by Cyt *c* present in the sera. However, the mAbs used for capture of rat Cyt *c* in the sandwich ELISA do not cross-react with the Cyts *c* of all the species tested. Furthermore, if that were the case, mouse serum would have increased the absorbance values in ELISA rather than decreasing them since mouse and rat Cyts *c* are identical [25].

The results shown in Fig. 1 were obtained employing a Cyt *c* concentration of 8 nM. In other experiments not shown inhibition was observed employing a range of Cyt *c* concentrations from 1 nM to 500 nM. The concentration of Cyt *c* in normal human serum has been reported to be 1.9 ng/ml or 0.15 nM [11], and may be elevated several hundred-fold in sera of patients with apoptosis-associated diseases [10, 12–17]. The real values may actually be higher than the measured values due to the effect of the serum inhibitory factor in the ELISA.

Partial purification of the inhibitory component from fetal bovine serum

Initial attempts to purify the inhibitory component by direct adsorption of horse serum or fetal bovine serum on

Cyt *c*-coupled Affi-Gel 10 resulted in multiple polypeptides eluting from the column as observed in SDS-PAGE (not shown). Furthermore, there was no specificity in the adsorption pattern in that the same bands were observed in the eluate from lysozyme-coupled Affi-Gel 10. (Lysozyme is an appropriate control since it is approximately the same size as Cyt *c* and has a similar pI.) Therefore, we proceeded to purify the inhibitory component from fetal bovine serum using a variety of methods and testing the fractions at each purification step for inhibition in sandwich ELISA. This approach included protein precipitation in saturated ammonium sulfate, ion exchange chromatography using DEAE, and gel filtration chromatography using Sephadex *G*-100 (see Materials and Methods). Inhibition of Cyt *c* detection in sandwich ELISA (Fig. 2, open circles) corresponded to the major fraction eluting from the gel filtration column, the last purification step (Fig. 2, closed circles).

The fraction corresponding to the highest A_{280} from the Sephadex *G*-100 column (fraction 40 from a parallel run) was subjected to *N*-terminal amino acid sequencing. The sequence of the first 10 residues was determined to be DTHKSEIAHR, the amino terminal sequence of the mature bovine serum albumin (BSA) polypeptide chain as searched employing BLAST [26].

BSA obtained from a commercial source (96–99% pure) was also found to have an inhibitory effect on Cyt *c* detection in the sandwich ELISA (Fig. 3, open circles). As was the case for serum, the inhibition by this preparation of BSA was observed whether mAb 1G1 or mAb 2G8 was used to capture rat Cyt *c*. The lowest concentration of the 96–99% pure BSA that resulted in complete inhibition of Cyt *c* detection in ELISA represents a molar ratio of approximately 1:1000 (Cyt *c*:BSA) suggesting that the inhibitory factor is a minor component in this BSA preparation.

We also tested a more pure form of BSA (>99%, essentially fatty acid free) obtained from the same supplier and found that it was much less inhibitory than the 96–99% pure BSA (Fig. 3, closed circles). This indicates that the inhibitory effect is due either to a minor component associated with BSA in the less pure preparation, such as a lipid, or to a contaminant that co-purifies with BSA.

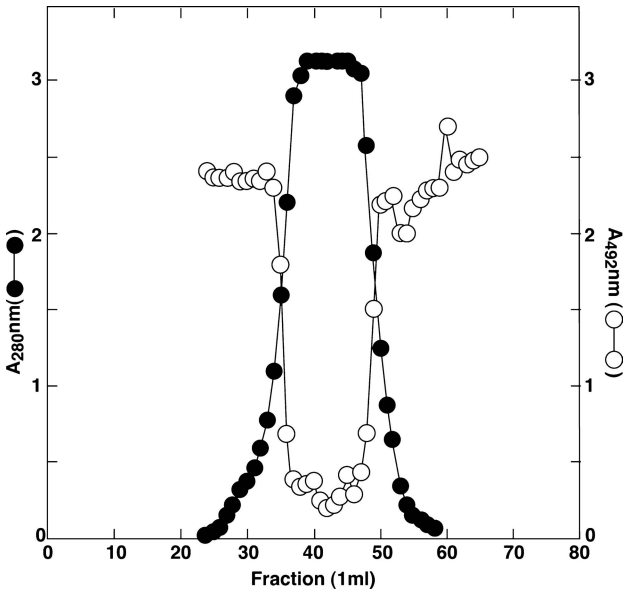

Fig. 2 Partial purification of the factor in fetal bovine serum that inhibits the detection of rat Cyt *c* in sandwich ELISA. Total protein ($A_{280\ nm}$) and effect on rat Cyt *c* detection in sandwich ELISA ($A_{492\ nm}$) are plotted for each fraction. The protein-containing fractions (closed circles) from the last purification step, gel filtration chromatography on Sephadex *G*-100, correspond to the inhibitory fractions (open circles)

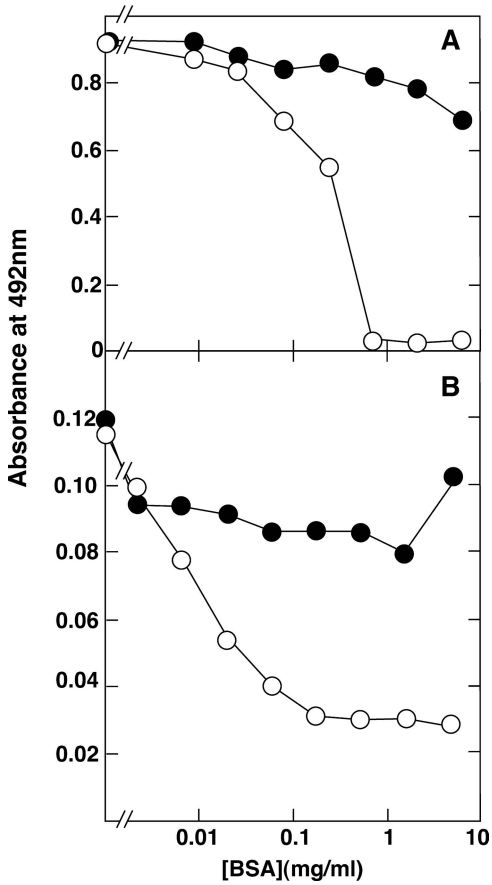

Fig. 3 BSA, 96–99% pure (○), inhibits the detection of rat Cyt *c* in sandwich ELISA much more effectively than the fatty acid-free form (>99%, ●). This difference was observed whether (A) mAb 1G1 was used for capture and mAb 2G8-HRP was used for detection or (B) mAb 2G8 was used for capture and mAb 1G1-HRP was used for detection. The values plotted represent the averages of triplicate readings with an average S.D. = 0.058

Characterization of the inhibitory mechanism

We observed no inhibition by serum or BSA (96–99% pure) if either were incubated with the capture antibody and then

Table 1 Inhibition by BSA (96–99% pure) of rat Cyt *c* detection in sandwich ELISA was eliminated by pre-adsorption on horse Cyt *c*-Affi-Gel 10 but not on Lipidex

Inhibitor	A492 nm
No BSA	0.625 ± 0.088
BSA[a] (96–99% pure) adsorbed on:	
Lysozyme coupled Affi-Gel	0.026 ± 0.003
Cyt *c* coupled Affi-Gel	0.622 ± 0.005
Lipidex	0.104 ± 0.019
Sephadex	0.093 ± 0.026

[a]BSA (96–99% pure) was tested at a concentration of 0.5 mg/ml.

Table 2 Inhibitory activity adsorbed on Cyt *c*-Affi-Gel 10 is retained following acid elution

Eluant of:	$A_{492\ nm}$ in sandwich ELISA for rat Cyt *c*
BSA (96–99% pure) adsorbed on Cyt *c*[a]	0. 075 ± 0.004
BSA (96–99% pure) adsorbed on lysozyme	0.250 ± 0.004
BSA (>99% pure) adsorbed on Cyt *c*	0.225 ± 0.040
BSA(>99% pure) adsorbed on lysozyme	0.235 ± 0.004

[a]Eluates were diluted 1:3 for the data reported. Further dilutions yielded less inhibition.

removed from the assay plate before the addition of Cyt *c* (results not shown). This indicates that the inhibitory component did not bind the antibody and block the capture of Cyt *c*.

Since BSA is a well-known lipid carrier we used Lipidex 1000 to adsorb lipids from BSA (96–99% pure) [23]. As shown in Table 1, this treatment failed to remove the inhibitory component indicating that it is probably not a lipid. The effect of the effluent on rat Cyt *c* detection in sandwich ELISA was essentially the same as the effluent from Sephadex *G*-25. The extensive dialysis used in the purification of the inhibitory fraction containing BSA (Fig. 2) would argue against some other small molecule inhibitor that was not bound to a larger molecular weight component in this material.

To determine if the inhibitory component bound Cyt *c*, horse Cyt *c* coupled to Affi-Gel was tested for its ability to remove the inhibitory component from BSA (96–99% pure). As shown in Table 1, following passage through a 1.5 ml column of Cyt *c* coupled Affi-Gel, there was very little inhibition remaining in the BSA preparation. In contrast, incubation with lysozyme failed to remove the inhibitory component. From this experiment we conclude that the inhibitory component binds Cyt *c*. Furthermore, the acid eluate, following lyophilization and neutralization, was found to contain the inhibitory component (Table 2).

Identification of the inhibitory factor in bovine serum

Proteins adsorbed from 96–99% pure BSA by the Cyt *c*-coupled Affi-Gel were eluted in 0.5 M acetic acid, lyophilized, and examined in SDS-PAGE. Although comparable small amounts of BSA from both the 99% pure and 96–99% pure preparations did adsorb to both the Cyt *c* and lysozyme coupled Affi-Gel columns, possibly due to electrostatic interactions between the negatively charged BSA and positively charged adsorbants (Fig. 4, arrow), there was a protein band at approximately 44 kD that was specifically adsorbed to the Cyt *c* coupled Affi-Gel column (Fig. 4(A), lane 2, arrowhead). This component was not adsorbed from >99% pure BSA by either lysozyme (Fig. 4(A), lane 3) or Cyt *c* (Fig. 4(A), lane 4) and was not adsorbed by

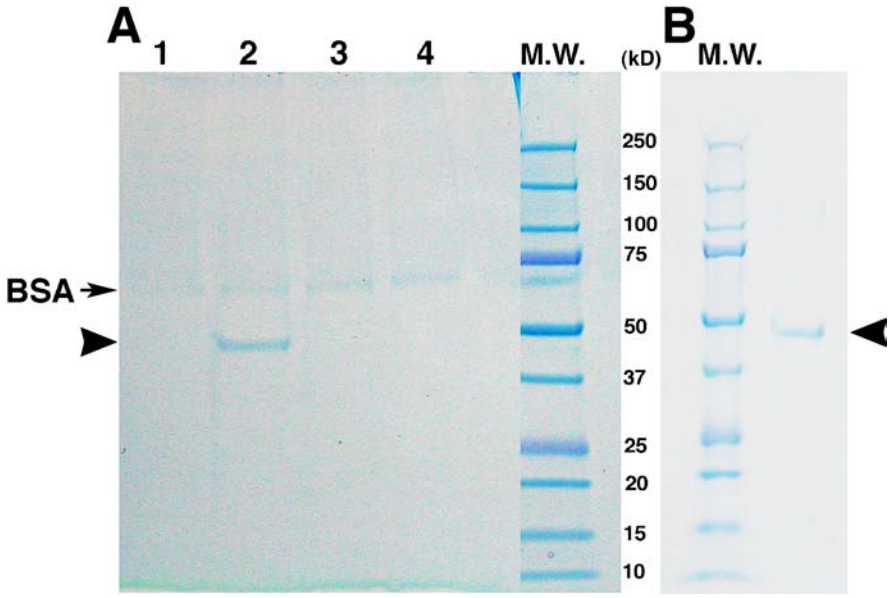

Fig. 4 SDS-PAGE of polypeptides once adsorbed from BSA onto Cyt *c*-Affi-Gel 10 or lysozyme-Affi-Gel 10 and eluted in 0.5 M acetic acid (A) or twice adsorbed onto Cyt *c*-Affi-Gel 10 and eluted (B). Lane 1, 96–99% pure BSA adsorbed on lysozyme; lane 2, 96–99% pure BSA adsorbed on Cyt *c*; lane 3,>99% BSA adsorbed on lysozyme; lane 4, 99% BSA adsorbed on Cyt *c*; m.w., molecular weight markers. BSA was identified by MALDI-TOF MS

lysozyme from 96–99% pure BSA (Fig. 4(A), lane 1). A second adsorption on Cyt *c* of the eluate from 96–99% pure BSA initially adsorbed on and eluted from Cyt *c* allowed for further enrichment of the 44 kD protein relative to BSA (Fig. 4(B), arrowhead). This isolate had similar inhibitory activity as the initial eluate (A492 nm $= 0.059 \pm 0.008$ at the same dilution tested, see Table 2).

Mass spectrometry analysis of tryptic peptides of the 44 kD polypeptide excised from the polyacrylamide gel (Fig. 4, lane 2) identified it as bovine leucine-rich alpha-2-glycoprotein-1 (LRα2GP1; NCBI BLAST gi/61878169) (Table 3). The tryptic peptides observed in MALDI-TOF MS encompass 42% of the length of the polypeptide chain predicted from the genomic sequence. The MASCOT score (www.matrixscience.com) of 193 is statistically significant (for scores >68, $p < 0.05$). MS/MS analysis of nine peptides yielded a cumulative ions score of 238. MALDI-TOF analysis of tryptic peptides from the total eluate from the Cyt *c*-Affi-Gel column also yielded a statistically significant score of 105 (for scores >47, $p < 0.05$) with 42% polypeptide coverage.

No peptide sequences upstream of residue 82 in the predicted amino acid sequence were observed (Table 3). It would appear that the mature polypeptide that we isolated derives from enzymatic cleavage of a precursor at the NEC1/NEC2 (proprotein convertases 1 and 2) cleavage site, carboxyl terminal to the sequence "KR" at residues 80–81 Although cDNA analysis of human and mouse LRα2GP1 predicted the amino terminus at position 85, not at position 82 as we observed, that methodology may not have allowed for isolation of full-length cDNA encompassing the amino terminal segment [27]. The sequence we isolated from bovine serum albumin initiates 37 amino acid residues upstream from human LRα2GP1 that was isolated from human serum two decades ago [28]. The higher molecular weight of the observed polypeptide versus the predicted polypeptide (residues 82–430) is approximately 9 kD consistent with glycosylation. There are 8 leucine-rich repeats in bovine LR2αGP1 with the consensus sequence "LxLxxNxL" that is shared by other leucine-rich proteins [29].

Identification of the inhibitory factor in human serum

We also isolated the inhibitory factor from human serum using a purification scheme similar to that followed for bovine LR2αGP1, including adsorption on Cyt *c*-Affi-Gel 10. (Unlike 96–99% BSA, we did not observe the inhibitory factor in 96–99% human serum albumin that we obtained from the same commercial source.) In sandwich ELISA the acid eluate (lyophilized and neutralized) inhibited detection of rat Cyt *c* ($A_{492\ nm} = 0.352 \pm 0.07$ in the absence of the eluate and $A_{492\ nm} = 0.079 \pm 0.015$ in the presence of a 1–10 dilution of the lyophilized eluate in PBS, further dilution yielded less inhibition).

Two bands were observed in SDS-PAGE (Fig. 5). The major band at 50 kD was identified by MALDI-TOF MS as human LRα2GP1 (Table 4; NCBI BLAST gi/72059) and the minor band near 70 kD was identified as hemopexin. The MASCOT score for LRα2GP1 was 142 (for scores > 69, $p < 0.05$) with 57% sequence coverage. MS/MS analysis of

Table 3 MALDI-TOF analysis of the bovine inhibitory factor identifies it as LRα2GP1

Residue numbers	Observed M.W.[a]	Predicted M.W	Amino acid sequence
82–93[b]	1376.68	1376.64	EATMSSQNPERK
194–202	1053.68	1053.66	FLLPVPQLK
203–208	715.42	715.42	VLDLTR
209–220	1354.7	1354.7	NSLTGLFPGFFR
221–231	1173.66	1173.68	VSAALCTLVLK
237–247	1299.7	1299.7	FLEASWLHGLK
293–299	797.44	797.44	GPLNLER
300–306	837.45	837.45	LHLEGNR
300–320	2281.27	2281.29	LHLEGNRLQVLGEGLLAPQPK
307–320	1461.86	1461.86	LQVLGEGLLAPQPK
321–330	1322.68	1322.71	LRYLFLNDNR
323–330	1053.54	1053.52	YLFLNDNR
331–340	961.54	961.53	LASVAAGAFR
345–368	2617.35	2617.37	LDMLDLSNNLLTTVPTGLWTSLGK (Oxid. M)
372–395	2880.35	2880.35	NLKDGFDISNNPWICDQNLADLYR
375–395	2525.12	2525.13	DGFDISNNPWICDQNLADLYR
396–403	972.5	972.5	WLVANENK
412–430	1922.95	1922.95	CAGPEALKGQTLLAAAESH
420–430	1096.55	1096.55	GQTLLAAAESH

[a]M.W., molecular weight.

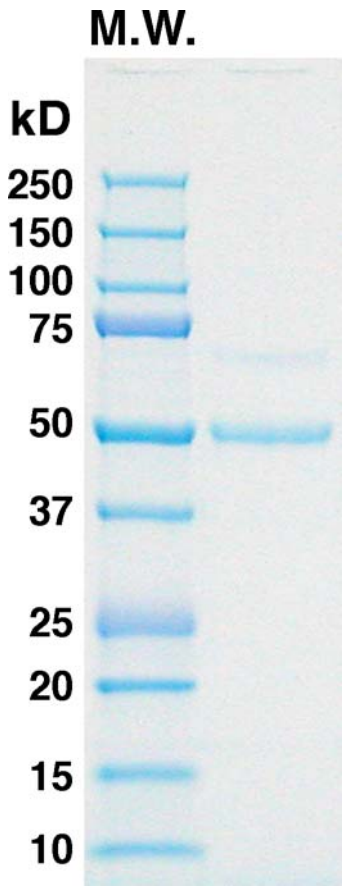

Fig. 5 SDS-PAGE of the human serum inhibitory factor following acid elution from Cyt *c*-Affi-Gel 10. The minor band near 70 kD was identified by MALDI-TOF MS as hemopexin which apparently bound some Cyt *c* molecules, possibly denatured during previous acid elution. The amount of eluate applied to the gel is approximately equal to that contained in 0.7 ml serum

two peptides (ALGHLDLSGNR and LHLEGNKLQVLGK) yielded significant individual ions scores of 52 and 67, respectively (for scores >47, $p < 0.05$). Hemopexin may have been present as a minor contaminant in the preparation due to adsorption to some denatured Cyt *c* molecules on the Affi-Gel column. In native Cyt *c* the heme is buried except for an edge [30], and is not accessible for binding hemopexin (R. Jemmerson and E. Margoliash, unpublished observation). Denaturation of some Cyt *c* molecules on the adsorption column may have occurred during prior acid elutions. Adsorption of a separate preparation of the inhibitory factor on a freshly prepared Cyt *c*-Affi-Gel column that was not previously acid washed enriched for the 50 kD band, while some Cyt *c* molecules not covalently bound to the Affi-Gel also eluted during the acid wash (not shown).

Discussion

Based on several clinical trials, Cyt *c* has been implicated as a valuable serum marker to measure excessive apoptotic activity in an individual [10, 12–17]. An increase in serum Cyt *c* appears to be a prognostic indicator of apoptotic disease progression. Therefore, it is important that the sensitivity for Cyt *c* detection be optimized. The sandwich ELISA is a convenient method to quantify proteins and has been used in most of the clinical trials to detect Cyt *c* in serum. However, antibody-based detection of Cyt *c* such as in sandwich ELISA may not be optimal in the presence of serum. We have shown that a component in serum binds Cyt *c* and inhibits its ability to be recognized by antibodies, thus decreasing the sensitivity of Cyt *c* detection in antibody-based assays such as sandwich ELISA. This component was present in the sera of all four species that were tested including human, cow, horse, and mouse.

Since during the course of purification of this component from fetal bovine serum we followed the inhibition of each isolated fraction, it would appear that the factor we isolated is the major, if not only, component in serum that interferes with the detection of Cyt *c* in sandwich ELISA. However, we cannot eliminate the possibility that other such factors, perhaps in lower concentration, may exist.

Although the inhibitory factor we isolated from fetal bovine serum co-purified with BSA, since the inhibitory effect was observed with 96–99% pure BSA but not observed with >99% pure BSA, the inhibitor is not albumin itself. Serum albumin is a well-known lipid binding protein that, in particular, binds fatty acids [31], and these are essentially absent in the >99% pure preparation. Cyt *c* is also known to bind fatty acids as well as other lipids, including phospholipids [32], Binding of these small molecules causes a conformational change in Cyt *c* that affects antibody recognition [33]. It was possible that lipids could have been transferred from the less pure BSA to Cyt *c*. However, passage of the 96–99% pure BSA through a Lipidex 1000 column, which effectively removes lipids from proteins, failed to remove the inhibitory activity.

By adsorption of the 96–99% pure BSA on Cyt *c* coupled to Affi-Gel 10, the inhibitory component was removed and shown by SDS-PAGE to be a 44 kD protein. From mass spectrometry analyses it was identified as bovine LRα2GP1. We found that the human homolog of this factor (50 kD) was not present in the 96–99% commercial preparation of human serum albumin but that it could be purified from serum employing a similar procedure as was used to isolate the bovine protein.

These findings may lead to modifications in the sandwich ELISA allowing for the quantification of total serum Cyt *c*. For example, a competitive ligand might be added to serum to displace any LRα2GP1-bound Cyt *c*. Alternatively, a mAb against LRα2GP1 and another mAb against Cyt *c* may be used in combination to capture and detect Cyt *c* complexed to LRα2GP1.

Table 4 MALDI-TOF analysis of the human inhibitory factor identifies it as LRα2GP1

Residue numbers	Observed M.W.[a]	Predicted M.W.	Amino acid sequence
42–47[b]	823.36	823.3647	DCQVFR
94–119	2958.6336	2958.5926	LQELHLSSNGLESLSPEFLRPVPQLR
120–125	715.4191	715.4228	VLDLTR
149–164	1893.0097	1892.9995	ENQLEVLEVSWLHGLK
165–175	1151.6091	1151.6047	ALGHLDLSGNR
165–177	1420.7893	1420.7898	ALGHLDLSGNRLR
192–209	2036.0951	2036.0789	TLDLGENQLETLPPDLLR
192–216	2829.5572	2829.5236	TLDLGENQLETLPPDLLRGPLQLER
210–216	811.4542	811.4552	GPLQLER
217–229	1447.8298	1447.851	LHLEGNKLQVLGK
230–239	1178.6654	1178.6659	DLLLPQPDLR
230–247	2128.1553	2128.168	DLLLPQPDLRYLFLNGNK
240–247	967.5126	967.5127	YLFLNGNK
240–250	1307.7361	1307.7349	YLFLNGNKLAR
251–260	988.5475	988.5453	VAAGAFQGLR
292–312	2484.1133	2484.1015	DGFDISGNPWICDQNLSDLYR
313–318	772.4123	772.4232	WLQAQK
329-345	1711.9338	1711.929	CAGPEAVKGQTLLAVAK

[a]M.W., molecular weight.

[b]Residue 42 is equivalent to residue 7 in the reported protein sequence [28].

LRα2GP1 is a novel ligand for Cyt *c*. The observation of Cyt *c* in normal serum [10–17] and its binding to LRα2GP1 suggest that extracellular Cyt *c* and the association between these two molecules may have physiological relevance. Cyt *c* could serve as an adaptor altering the activity of LRα2GP1. Modulation of the activity of another protein, Apaf-1, by Cyt *c* is well known [3–5]. In binding Apaf-1 Cyt *c* acts as a co-factor in the activation of caspase-9 allowing Apaf-1 to undergo a conformational change [5]. It may be relevant that LRα2GP1 is produced by neutrophils [27] and neutrophils have been implicated as a key effector cell in Cyt *c*-induced arthritis [11]. Since the function of LRα2GP1 itself is not known it is difficult to speculate further on the actual effect of Cyt *c* binding on LRα2GP1 function and *vice versa*. Whatever that effect may be, the present study does extend the remarkable list of Cyt *c* ligands in apoptosis that have been reported (reviewed in ref. [2]).

Conflict of interest disclosure The University of Minnesota and R. Jemmerson have license agreements with companies for the use of several mAbs reported herein to detect and quantify Cyt *c*.

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