Characterization of a thioredoxin-related surface protein

Michael F. DEAN,*† Harry MARTIN† and Paul A. SANSOM*

*Biochemistry Division, Kennedy Institute of Rheumatology, Bute Gardens, Hammersmith, London W6 7DW, U.K.

and †Laboratory of Protein Structure, N.I.M.R., Mill Hill, London NW7 1AA, U.K.

A surface-associated sulphhydryl (thiol) protein (SASP) constitutively present in most nucleated cells was purified from human THP-1 monocytes and rat C6 glioma cells. The human protein was similar in mass and isoelectric point and had the same N-terminal amino acid sequence to adult T-cell leukaemia-derived factor (ADF), a growth factor secreted by human lymphoid cells which is able to induce increased expression of interleukin-2 receptors. A further internal amino acid sequence, determined following cleavage of human SASP with cyanogen bromide, was also identical to the corresponding sequence deduced for ADF. Samples of SASP were able to reductively depolymerize human immunoglobulin, a property shared with thioredoxin, a ubiqui-

tous protein, almost identical to ADF, with an essential function in many thiol-dependent reducing reactions. Furthermore, SASP purified from rat C6 glioma cells had an identical N-terminal amino acid sequence to that deduced for rat liver thioredoxin, showing that they were both members of the same family of proteins. The use of membrane-impermeable thiol reagents indicated that SASP was predominantly a cell-surface protein, and was not normally secreted. This SASP protein appeared to be a surface-associated form of thioredoxin that was constitutively present in a wide range of cells and was related to ADF, a secreted form of the same protein.

INTRODUCTION

Thioredoxin is a small thiol-containing protein widely distributed in bacteria, plants, mammals and humans (Holmgren, 1968; Söderberg et al., 1978; Holmgren, 1989; Wedel et al., 1992), where the gene is located on the short arm of chromosome 3 (Lafage-Pochitaloff-Huvalle et al., 1989). The protein contains a redox-active disulphide which has a crucial role in the catalysis of a wide range of oxidation and reduction reactions such as the refolding of disulphide-containing proteins (Pigiet and Schuster, 1986), the stabilization of glucocorticoid receptors (Grippo et al., 1985) and the depolymerization of insulin (Holmgren, 1979). A number of related proteins of similar size and highly similar in structure to thioredoxin have been described. These include the adult T cell leukaemia-derived factor (ADF) which has cytokine-like activities, can act as an autocrine growth factor by up-regulating expression of interleukin (IL)-2 receptors and can synergize with IL-1 and IL-2 (Tagaya et al., 1987, 1988, 1989; Wakasugi et al., 1990). Another related protein is human eosinophil cytotoxicity-enhancing factor (ECEP) which is secreted by U937 monocytes and greatly enhances antibody-dependent cytotoxicity (Silberstein et al., 1987, 1989; Balcewicz-Sablinska et al., 1991). Cytokine activity is also present in a growth factor secreted by the Epstein-Barr virus-transformed B-cell line 3B6 that appears to be almost identical to ADF (Wakasugi et al., 1990).

We recently described a surface-associated thiol (sulphydryl) protein (SASP) (Martin and Dean, 1991) that contained free thiol groups, could be readily labelled with [¹⁴C]iodoacetamide and was present on all nucleated cells. The SASP purified from human THP-1 monocytes was found to have an identical *M_r* and N-terminal amino acid sequence to both human thioredoxin and ADF protein. We have now investigated further the relationship between SASP, thioredoxin and other related proteins by determining additional amino acid sequences of human SASP and

by comparing the N-terminal sequence of SASP purified from rat glial cells with that deduced previously for rat thioredoxin. In addition we have shown that a large proportion of SASP is present on external cell membranes, that it has similar reducing properties to thioredoxin and is able to reductively depolymerize IgG. These data indicate that SASP is probably a surface-associated form of the thioredoxin family of proteins.

MATERIALS AND METHODS

Materials

Tissue culture media, supplements and Dulbecco's PBS were supplied by Flow Laboratories, Rickmansworth, Herts, U.K. and serum by Imperial Laboratories, Salisbury, Wilts, U.K. Immobilon-P membrane was purchased from Millipore, U.K., Bio-lyte carrier ampholyte, 3-10 and protein assay reagents were from Bio-Rad Laboratories, U.K. Labelled molecular weight markers, Amplify, enhanced chemiluminescence (ECL) reagents and [¹⁴C]iodoacetamide were obtained from Amersham, U.K. All other reagents were from Sigma, U.K.

Purification of SASP

Cells were harvested by centrifugation, washed twice with ice-cold PBS and suspended for 15 min in PBS (10⁸ cells/ml) containing 5 µCi of [¹⁴C]iodoacetamide. Untreated thiol groups were blocked by adding 200 µl of 1.0 M unlabelled iodoacetamide, and the cells were washed in 10 ml of PBS at 4 °C, and then pelleted and solubilized for 15 min in 0.5 ml of 0.5% (w/v) CHAPS in water containing 20 mM dithiothreitol, 5 mM iodoacetamide, 1 mM phenylmethylsulphonyl fluoride and 2 mM EDTA (solution A). The pellet was re-extracted with a further 0.5 ml of CHAPS solution, four preparations were pooled, an equal volume of water was added and precipitated proteins were

Abbreviations used: SASP, surface-associated thiol (sulphydryl) protein; ADF, adult T cell leukaemia-derived factor; IL, interleukin; ECEP, eosinophil cytotoxicity-enhancing factor; ECL, enhanced chemiluminescence; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); pCMPSA, *p*-chloromercuriphenylsulphonic acid; 6-IAF, 6-iodoacetamidofluorescein; HRP, horseradish peroxidase; FCS, foetal calf serum; PHA, phytohaemagglutinin; TNFα, tumour necrosis factor-α.

† To whom correspondence should be addressed.

removed by centrifugation. The supernatant solution was diluted to 40 ml with water, 2 ml of Biolyte 3-10 ampholyte was added and the sample subjected to preparative isoelectric focusing using a Bio-Rad Rotophor at 12 W constant power until a stable voltage was reached (about 950 V after 4 h). Fractions of 2 ml were collected and those containing radiolabelled protein were pooled, concentrated and ampholytes removed by centrifugation through an Amicon Centricon membrane with a molecular mass cut-off of 10 kDa for 40 min at 4500 *g*. The concentrate was loaded onto a column of Sephadex G-75 fine (1.5 × 75 cm) which was packed and eluted in a 1:10 dilution of PBS/0.5% CHAPS in solution A. Fractions of 1.5 ml were collected, the radiolabelled peaks concentrated, desalted by centrifugation through an Amicon membrane and stored at -20 °C.

Sequencing and cleavage

Gradient polyacrylamide gels (10–20%) were pre-electrophoresed for 1 h at 8 mA constant current then loaded with 5 µg samples of purified SASP per track. After electrophoresis for 3 h at 20 mA constant current the gels were wet blotted onto 'Immobilon-P' membrane, which was prepared by dipping it for 2 s into 100% methanol, washing in water and soaking in transfer buffer (10 mM CHAPS in 10% methanol, adjusted to pH 11 with 4 M NaOH). Blotting was carried out for 18 h at 4 °C at a constant current of 80 mA, the membrane was stained (0.1% Coomassie Blue in 50% methanol/10% acetic acid), washed in water, and dried in air. Unblocked N-terminal amino acid sequences were determined by Glynis Robinson of the protein sequencing service, Trinity College, Dublin, using an applied Biosystems 4774 pulsed liquid synthesizer with an on-line 120A PTH analyser.

Further internal sequences were determined following indirect cleavage with CNBr using a protocol similar to that described by Zingde et al. (1986). For this segments containing SASP were cut from SDS gels after electrophoresis, diced and freeze dried. They were then left in contact with the vapour generated by the addition of 300 mg of CNBr to 8 ml of 70% formic acid for 72 h in a sealed 150 ml Sterilin jar. CNBr was removed by adding 4 × 100 µl aliquots of water, and freeze-drying the gel. The remaining proteins were then extracted for 24 h in 100 µl of sample buffer, subjected to SDS/PAGE, and sequenced as described above. In some experiments existing N-terminal groups were blocked by succinylation after purified SASP had been wet blotted onto Immobilon membranes and new N-terminals then generated by CNBr cleavage of the transferred protein. For this pieces of Immobilon-P membrane containing the transferred protein were equilibrated in 500 ml of 0.2 M sodium borate in 10% methanol, pH 8.5, and 500 mg of succinic anhydride added in batches of 50 mg at intervals of 10 min. The pH was maintained at 8.5 with 1 M NaOH, and succinylation was allowed to proceed for 2 h after the final addition. The membrane was then washed three times in 10% ethanol and subjected to cleavage in CNBr vapour as described above.

Surface labelling

THP-1 cells were suspended at a concentration of 1.25×10^7 cells in 10 ml of PBS containing 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) or 20 mM *p*-chloromercuriphenylsulphonic acid (pCMPSA) or in PBS alone for 30 min at 4 °C. The cells were then washed with 2 × 10 ml of PBS, resuspended in a further 1 ml, and surface thiol groups labelled by adding 100 µl of a solution of 6-iodoacetamidofluorescein (6-IAF) (0.45 mg/ml of water) for 10 min at 4 °C. Any remaining free thiol groups were blocked by adding 100 µl of a solution of 1 M iodoacetamide in

water, the cells again washed with 2 × 10 ml aliquots of PBS and solubilized in 0.5 ml of 0.5% (w/v) CHAPS in solution A. Labelled proteins were separated by SDS/PAGE on a 10–20% gradient gel and then wet blotted onto Immobilon-P membranes as described above. The membranes were blocked for 1 h with solution B (150 mmol NaCl, 10 mmol P_i containing 5% (w/v) milk powder and 0.1% (v/v) Tween 20) and incubated with a 1:1000 dilution of rabbit anti-fluorescein, horseradish peroxidase (HRP) conjugate (Amersham NIF 813) in solution B for 1 h. After three further washings, the blots were incubated with ECL reagent for 1 min, drained, covered in Saranwrap and exposed to XAR-5 film for between 0.5 and 10 min.

IgG reduction

Low molecular mass fragments were removed from a sample of human IgG by gel chromatography on a 60 ml bed volume column of Sephadex G-200 and the IgG fractions that eluted immediately after V_0 were pooled to give a preparation containing 4.4 mg/ml of protein. Proteins were extracted from 1.3×10^8 THP-1 cells in 0.5% CHAPS without prior labelling of free thiol groups and spiked with a sample of labelled SASP (10000 c.p.m.) as a marker. The combined extracts were purified by dilution in water, centrifugation and preparative isoelectric focusing as described above, concentrated by centrifugation through a Centricon membrane and activated by incubating for 1 h at room temperature in 50 mM dithiothreitol in activation buffer (80 mM Hepes, 10 mM EDTA, pH 7.5). The dithiothreitol was removed by centrifugation through a Centricon membrane with a cut-off of 10 kDa, washed twice with 2 ml of SASP activation buffer, concentrated after each wash and reduced to a final volume of 500 µl. Samples containing 20 µl of this activated-SASP preparation were mixed with an equal volume of IgG and left for 1 h at room temperature prior to separation by SDS/PAGE. Control samples of IgG were left for 1 h in contact with either 50 mM dithiothreitol or activation buffer containing 50 mM dithiothreitol that had been centrifuged through a Centricon membrane and washed twice. All samples were then analysed by SDS/PAGE on a 5–20% gradient gel under non-reducing conditions.

Electrophoresis

SDS/PAGE was carried out using the method of Laemmli on 5–20% or 10–20% gradient slab gels (Laemmli, 1970) with labelled low molecular mass standards in the range 46–2.3 kDa as markers. Fluorography was carried out on gels that had been impregnated with Amplify for 30 min, dried and kept in contact with Kodak XAR-5 film for 2–7 days at -70 °C. Analytical isoelectric focusing was carried out using pre-cast 1 mM thick 5% polyacrylamide gels and 2.2% (w/v) ampholine in the pH range 3.5–9.5 (Pharmacia). After focusing for 4 h at 1 W/cm the gels were fixed in 10% trichloroacetic acid and proteins were visualized either by staining with Coomassie Blue or by fluorography.

Stimulation with phytohaemagglutinin (PHA)

Rapidly growing THP-1 cells were harvested, transferred into antibiotic-free RPMI 1640 medium containing 5% foetal calf serum (FCS) and 1 µg/ml PHA and aliquoted into 25 cm² flasks (10^6 cells in 5 ml of medium). Control flasks were set up without PHA. After 3 days the cells were collected by centrifugation, extracted in 0.5 ml of 0.5% (w/v) CHAPS and labelled with 6-IAF. The conditioned media were concentrated to 0.5 ml by centrifugation through an Amicon membrane, cut-off 10 kDa, and aliquots from each extract containing 50 µg of protein were

subjected to SDS/PAGE. SASP was visualized after Western blotting by reaction with HRP anti-fluorescein antibody and ECL as described above.

Cell proliferation

U937 cells (2×10^5 /ml) in RPMI 1640 medium containing 10% FCS and 100 units/ml interferon- γ were dispensed into 96-well microtitre plates in 100 μ l aliquots. The plates were then incubated overnight at 37 °C in a humidified 5% CO₂ atmosphere and 10 μ l of a stock solution of either 500 ng/ml or 50 ng/ml tumour necrosis factor- α (TNF- α) in the above medium containing 10 μ g/ml actinomycin D added to some of the wells in quadruplicate replicates. To others, 10 μ l of a 50 mg/ml solution of SASP prepared without the addition of iodoacetamide was added either alone or in combination with TNF- α and 10 μ l of medium added to control, untreated wells. After a further 24 h of incubation cell proliferation was assayed by the method of Mosmann (1983). For this 15 μ l of a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide ('MTT') (5 mg/ml in PBS) was added to each well for 4 h at 37 °C. The formazan product was solubilized by adding 100 μ l of 0.04 M HCl in isopropanol for 1 h with intermittent shaking and read at 600 nm on a Bio-Tek instruments microplate reader.

Assays

Protein concentrations were determined with the Bio-Rad reagent (Bradford, 1976) using bovine γ -globulin as a standard. Conductivity of samples after gel chromatography or isoelectric focusing were determined with a Radiometer CDM 83 conductivity meter. Radioactivity was determined using 0.5 ml samples added to 3 ml of Optiphase scintillation cocktail (LKB) and counted in a Wallac 1410 spectrometer (Pharmacia).

RESULTS

Purification

Under the conditions used routinely for these experiments almost 50% of the radioactivity added to each batch of cells was extracted in the CHAPS solution. More than half of this was insoluble after dilution with water and was discarded prior to

Figure 2 Separation of SASP on Sephadex G-75

The fractions containing SASP proteins separated by isoelectric focusing were concentrated and further purified by gel filtration chromatography on Sephadex G-75. Fractions of 1.5 ml were collected and assayed for radioactivity (■) and protein (○).

Figure 3 Purification of SASP

(a) Samples of labelled protein from each stage of purification were analysed by SDS/PAGE on a 10–20% gradient gel under reducing conditions and stained with Coomassie Blue. (b) The same gel was dried and the radiolabelled proteins visualized by fluorography. Lanes: 1 = molecular mass markers, 2 = CHAPS extract, 3 = proteins after isoelectric focusing, 4 = proteins after chromatography on Sephadex G-75.

Figure 1 Isoelectric focusing of labelled proteins from THP-1 cells

The soluble proteins remaining after extraction of 2×10^8 [¹⁴C]iodoacetamide-labelled cells in 0.5% CHAPS in solution A and dilution with water were subjected to preparative isoelectric focusing until a constant voltage was reached. The separated proteins were collected in 2 ml fractions and each fraction monitored for radioactivity (■), protein (●) and pH (□).

focusing. After focusing, a single sharp peak of radioactivity with a pI of 4.8 was separated from a general background of labelled material that was present in the more acidic fractions preceding the peak (Figure 1). Other proteins precipitated during focusing, so that only one major component corresponding to the peak of radioactivity was present after this stage of the purification. This fraction was subjected to gel filtration on Sephadex G-75 after first being concentrated through a centricon membrane and was separated into two radioactive components (Figure 2) the larger of which with a K_{av} of 0.45 corresponded to

Figure 4 Two-dimensional electrophoresis of SASP

A sample of purified SASP protein was first separated by isoelectric focusing on precast 1 mm-thick 5% polyacrylamide gels containing 2.2% (w/v) Ampholine. A strip was then cut from the gel and subjected to SDS/PAGE on a 10–20% gradient gel. After electrophoresis for 4 h at 1 W/cm the gels were dried and subjected to fluorography.

(a) Human thioredoxin	MVKQIESKTAFQEALDAAGDK
SASP	(M)VKQIESKTAFQEALDAAGDK
(b) Human thioredoxin	IKPFFHSLSEKYSNVIFLEV
ADF protein	IKPFFHSLSEKYSNVIFLEV
SASP	IKPFFHSLSEKYSNVIFLEV
(c) Rat thioredoxin	MVKLIESKEAFQEALAAAGDK
Rat SASP	(M)VKLIESKEAFQEALAAAGDK K GLQL

Figure 5 Comparison of amino acid sequences

(a) The N-terminal sequence of SASP from THP-1 cells, designated by the single letter code, was compared with those of human thioredoxin (Forman-Kay, 1989) and ADF protein (Tagaya et al., 1989) after PAGE and wet blotting. (b) The sequence determined following CNBr cleavage of SASP at methionine residues was compared with that determined for the corresponding sequences for ADF protein (Tagaya et al., 1989) and thioredoxin (Forman-Kay, 1989). (c) The N-terminal sequence of SASP from rat C6 glial cells was compared with that of rat liver thioredoxin (Tonissen et al., 1989) after PAGE and wet blotting. In some cases where specific amino acids could not be assigned unambiguously, a possible alternative is given below the horizontal bar.

SASP and contained 0.5% of the initial radioactivity added to the cells.

Samples from each stage of the purification were analysed by SDS/PAGE under reducing conditions. The initial CHAPS extract contained more than 30 separate components only two of which were well labelled with [^{14}C]iodoacetamide (Figure 3). The heaviest degree of labelling was in the fastest moving band, which had an M_r of 11.5 kDa and which sometimes appeared as a doublet. The other heavily labelled band was much larger, with an M_r of 52 kDa. After focusing only the faster moving of the labelled bands could be detected and the number of other protein components present had been greatly reduced. The final purification step using gel filtration left only a single, detectable, labelled protein. This was confirmed by two-dimensional electrophoresis

in which samples of the purified protein were first separated by isoelectric focusing, then subjected to SDS/PAGE (Figure 4).

Sequencing

The N-terminal amino acid of purified, human SASP was not blocked, allowing the sequence of the first 20 amino acids to be determined in three separate analyses. This sequence was identical to those deduced from the cDNA sequences for both human thioredoxin and ADF protein except for the absence of an initial methionine at residue one (Figure 5a). In some analyses of SASP, however, methionine did appear as a strong secondary signal in addition to valine in cycle 1. The sequences deduced for human thioredoxin and ADF protein (Wollman et al., 1988; Tagaya et al., 1989) indicated that one or two additional internal methionine residues were also present in these proteins. When samples of SASP were treated with CNBr they too were cleaved but the fragments produced could not be cleanly separated on SDS/PAGE because of their similar sizes. When the existing N-termini were blocked by succinylation prior to cleavage, two distinct sequences were detected. One of these corresponded to that previously deduced for the original N-terminal and the other to a new sequence resulting from cleavage at additional internal methionine residues. This new sequence was identical to that deduced for the corresponding CNBr cleavage product of ADF protein and for the sequence obtained for recombinant human thioredoxin by Forman-Kay et al. (1989) (Figure 5b) but differed from the sequence published for human thioredoxin by Wollman et al. (1988) in having a lysine at residue 2 of the cleavage fragment in place of asparagine. In some cycles evidence of a third sequence was detected, indicating that there may have been a second internal methionine cleavage site.

An N-terminal amino acid sequence was also obtained for SASP purified from rat C6 glial cells by the same procedure as that used for THP-1 cells. This sequence was again almost identical with that deduced from a cDNA clone for rat liver thioredoxin except for the absence of methionine in cycle 1 (Figure 5c).

Blocking of surface thiol groups

When THP-1 cells were labelled under normal conditions at 4 °C with 6-IAF as an alternative to [^{14}C]iodoacetamide, a large number of thiol-containing proteins were detected after the cell extracts had been subjected to SDS/PAGE and ECL. These included a heavily labelled SASP component with an M_r of 11.5 kDa (Figure 6, lane 1). When surface thiol groups were first blocked by reacting them with either of the membrane impermeable reagents DTNB or pCMPSA before labelling, the number of bands was reduced considerably and the few that remained were much less heavily labelled than in control, unblocked cells (lanes 2 and 3). Among these weakly labelling proteins was SASP, very little of which was now present in a form still able to react with 6-IAF. After stimulation of THP-1 cells for 3 days with PHA the amount of labelled SASP increased noticeably and a small amount of SASP was detected in concentrated medium harvested from stimulated cells (results not shown). However, no SASP was detected in medium collected from control, unstimulated cells (lane 4).

Reduction of immunoglobulin

Samples of SASP were incubated with normal human IgG to determine if it possessed similar reducing properties to thio-

Figure 6 Surface labelling of THP-1 cells

Surface thiol groups were labelled with 6-IAF before and after blocking with DTNB or pCMPSA, separated by SDS/PAGE and wet blotted onto Immobilon membranes. The blots were then probed with an anti-fluorescein HRP-conjugated antibody incubated with ECL reagent for 1 min then exposed to Kodak XAR-film for 4 min. Lanes: 1 = control, unblocked cells, 2 = cells after reaction with DTNB, 3 = cells after reaction with pCMPSA, 4 = conditioned medium taken from unblocked cells.

Figure 7 Effect of SASP on IgG

A sample of human IgG (10 mg in 1 ml of PBS) was purified by chromatography on Sephadex G200 then analysed by SDS/PAGE on a 10–20% gradient gel under non-reducing conditions before and after incubation with SASP or 50 mM dithiothreitol for 1 h. Lanes: 1 = IgG alone, 2 = IgG + activation buffer, 3 = IgG after reduction with dithiothreitol, 4 = IgG after incubation with SASP, 5 = molecular mass markers.

redoxin and was able to depolymerize immunoglobulins. Un-reduced IgG was resolved into one major band on SDS/PAGE plus three smaller components, which may have been breakdown products or separated sub-classes (Figure 7, lane 1). After a sample had been mixed for 1 h with dithiothreitol, the amount of whole IgG present had decreased, due to reductive depolymerization and there was an accompanying increase in free heavy and light chains at 51 kDa and 22 kDa respectively (lane 3). A similar depolymerization pattern was seen when samples of IgG were incubated for 1 h with SASP (lane 4), but the control sample of IgG that had been incubated with activation buffer alone remained unreduced (lane 2).

Biological activity

When U937 monocytic cells were incubated with TNF α after sensitization with interferon- γ , their proliferative activity was reduced by 80% at a concentration of 50 ng/ml and by 50% at 5 ng/ml. In contrast SASP by itself had a stimulatory effect, increasing proliferation to 25% above control levels at

0.5 mg/ml. When SASP at this concentration was added with 50 ng/ml TNF α , proliferative activity was restored to only 5% below control levels and with 5 ng/ml TNF α was increased to 20% above normal levels.

DISCUSSION

The SASP purified from THP-1 cells had a M_r of 11.5 kDa and a pI of 4.8, values very close to those that had been determined previously for human thioredoxin of 11.68 kDa and pI 4.88 (Wollman et al., 1988). The highly homologous ADF protein secreted by human-T-cell-lymphotrophic-virus-1-transformed human T cells has an M_r of 12.7 kDa (Tagaya et al., 1989) and appears to be identical to the cytokine-like protein secreted by 3B6 B cells, with a pI of 5.0 (Wakasugi et al., 1990). The sequence of the first 20 N-terminal amino acids of SASP was identical to that deduced from the cDNA for human thioredoxin, except for the initial methionine residue (Wollman et al., 1988). In some analyses of SASP, methionine did however appear as a secondary signal, in addition to the valine residue in cycle 1 suggesting that it may be present in two isoforms. Similarly, two types of human thioredoxin have been reported previously, arising from sequence heterogeneity at the N-terminus (Forman-Kay et al., 1989). One of these forms has an N-terminal methionine suggesting that not all of the translation initiating methionine is removed during maturation of the protein. It is likely therefore that SASP behaves in a similar fashion.

In the sequences deduced previously for thioredoxin and ADF protein (Wollman et al., 1988; Tagaya et al., 1989) an internal methionine was present at residue 36, rendering these proteins susceptible to cleavage with CNBr. When SASP was subjected to CNBr treatment it too was cleaved and the new N-terminal sequence exposed was identical to that deduced previously for ADF protein by Forman-Kay et al. (1989) and Eklund et al. (1989) but differed from the sequence published by Wollman et al. (1988) in having a lysine residue at position 39 in place of asparagine. In addition, secondary signals detected during sequencing of our CNBr cleavage products, suggested that a second internal methionine may have been present and that the protein had also been cleaved at this point. Although the deduced sequence for thioredoxin does not contain any additional methionine, ADF protein does have a methionine at position 74 in place of threonine residue deduced for thioredoxin, so that SASP may again be similar to ADF protein rather than thioredoxin in this respect.

Although both ADF and the 3B6 proteins have been purified and sequenced only from human leucocytes, the sequence of thioredoxin has been determined for a variety of species including rabbit (Johnson et al., 1988), monkey (An and Wu, 1992) and rat (Tonissen et al., 1989). These sequences are all very similar but that determined for rat liver, although 89% identical to human thioredoxin, differs in having two substitutions near the N-terminus: leucine instead of valine at position 4 and glutamic acid instead of lysine at position 9. We purified SASP from rat C6 glial cells and found that its N-terminal sequence was identical to that deduced for rat liver thioredoxin, with identical substitutions at positions 4 and 9; further evidence that SASP is a form of thioredoxin.

A characteristic of thioredoxins is that they contain a highly conserved active redox amino acid sequence (Cys-Gly-Pro-Cys) which enables them to act as cofactors in a variety of important oxidation-reduction reactions (Tagaya et al., 1990). This sequence is also present in ADF protein (Tagaya et al., 1989) and is thought to play an essential role in redox-mediated activation of target cells by ADF. As a result of this redox potential human

thioredoxin has the capacity to reductively depolymerize IgM and release free heavy and light chains (Wollman et al., 1988). Although we have no direct evidence that SASP contains a conserved Cys-Gly-Pro-Cys sequence we did show that it was able to reductively depolymerize human IgG, and it is therefore highly likely that either this sequence, or the closely related Cys-Gly-His-Cys sequence, that has been described in the related phosphoinositide-specific phospholipase C enzyme (Bennett et al., 1988), may be present. In addition, SASP increased the rate of proliferation of U937 monocytic cells and protected them from the cytotoxicity induced by TNF- α . Both of these properties are characteristic of ADF protein (Wakasugi et al., 1990; Matsuda et al., 1991) and are attributed to its ability to alter the intracellular redox environment or to reduce a target protein present on cell membranes.

In rat hepatocytes thioredoxin is widely distributed throughout cells with high concentrations in the endoplasmic reticulum and Golgi cisternae (Rozell et al., 1988) and it may also be present at the surface of some mammalian cells (Stemme et al., 1985). Although ADF has been characterized primarily as a secreted protein it may also exist as a membrane-bound form (Matsuda et al., 1991). Another cytokine-like protein, ECEF, synthesized by U937 cells, can exist in three forms of 10, 13 and 14 kDa, each of which has an identical or highly similar N-terminal sequence to that of human thioredoxin (Balcewicz-Sablinska et al., 1991; Silberstein et al., 1993). This factor, which can enhance the cytotoxic effect of specific antibodies and can act as a dithiol reductase, is normally secreted as a 14 or 13 kDa protein, while the 10 kDa form remains mainly cell associated and appears to be located on the external surface of the plasma membrane. Earlier experiments with SASP have shown that it can be stripped from cells in acetate buffer at pH 3.5, it appears to be present in a mainly cell-surface associated form, and also it contains free thiol groups (Martin and Dean, 1991). In further experiments reported here we have found that when surface thiol groups on THP-1 cells were first blocked with membrane-impermeable thiol antagonists such as DTNB or pCMPSA, very little SASP remained in a form able to react with labelled iodoacetamide, confirming that most of it was present on the external cell surface. SASP therefore appears to be analogous to the surface form of ECEF in this respect.

Unlike most secreted proteins ADF and thioredoxin both lack an N-terminal hydrophobic signal sequence. Proteins which do not have this targeting sequence normally remain within the cytoplasm (Verner and Schatz, 1988) with few exceptions. Among these is the cytokine IL-1, the α and β forms of which both lack a signal sequence although they are processed to a mature form and secreted (Auron et al., 1984; March et al., 1985; Singer et al., 1988). Thioredoxin is not normally secreted by B and T cells unless they are stimulated with PHA (Rubartelli et al., 1992) and secretion is increased when transport along the normal secretory pathway is blocked with drugs such as brefeldin or monensin (Rubartelli and Sitia, 1991; Rubartelli et al., 1992). Enhancement of IL-1- β secretion has also been reported under similar conditions (Rubartelli et al., 1990) suggesting that each of these proteins may be secreted along similar but not identical alternative pathways.

Thioredoxin, ADF protein and SASP appear to be virtually identical molecules and SASP may therefore be considered as a surface-associated form of thioredoxin that is normally present on a wide variety of different cell types. ADF protein, however, appears to be mainly a secretory product, which is produced endogenously by HTLV-1 or EBV transformed cells but does not appear to be produced by normal mononuclear cells unless stimulated with PHA or phorbol 12-myristate 13-acetate

(Wakasugi et al., 1990). The surface-associated form of thioredoxin (SASP) in contrast is constitutively present on a range of normal cells (Martin and Dean, 1991). Synthesis of SASP by THP-1 cells could however be up-regulated by PHA, which also induces some secretion. It may be therefore that if ADF and SASP are forms of the same protein, which seems highly likely, that when synthesis is up-regulated due to viral transformation or other stimulus, the excess protein is not inserted into the membrane but is exported. Alternatively, surface-associated proteins may represent a transitory pool of thioredoxin awaiting release as a secretory protein when cells are subjected to an appropriate stimulus.

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