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Identification of a thioredoxin-related protein associated with plasma membranes

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A low molecular weight membrane associated sulphydryl protein was detected on a wide range of nucleated cells when [^{14}C]-iodoacetamide was used as a probe. This protein was extracted from THP-1 monocytes, purified to homogeneity and its isoelectric point, M_r and N-terminal amino acid sequence determined. These were shown to be almost identical to the corresponding values for both human thioredoxin and a Tac interleukin-2 receptor activator, indicating that the protein may be a member of this family and function as an essential growth factor.

Many proteins associated with plasma membranes contain sulphydryl groups and have important physiological functions. For example, surface sulphydryl groups are required by macrophages for endocytosis of immune-complexes [1]. The existence of a sulphydryl bearing "zipper" molecule on the surface of phagocytic cells has been postulated [2], while alkylation of free sulphydryl groups in hamster kidney cells showed that they were essential for cell adhesion [3]. Another small sulphydryl containing protein, thioredoxin, is widely distributed in bacteriophages, bacteria, plants and mammals [4-7]. Thioredoxin has a central role in a large number of dithiol-disulphide oxidation and reduction reactions and is located on the surface of some mammalian cells [8, 9]. A protein secreted by HTLV-1 transformed T-cells and highly homologous to thioredoxin can also be present in a membrane associated form. This protein (ADF) can act as an autocrine growth factor by upregulating expression of interleukin-2 receptors on T cells [10].

In this study we attempted to identify cell surface associated sulphydryl proteins by labelling them with [^{14}C]-iodoacetamide and found that a low molecular weight protein was the predominant component present on all nucleated cell types examined. This surface associated sulphydryl protein (SASP) was purified from human THP-1 monocytes and its N-terminal amino acid sequence determined. Comparison of the sequence with those deduced previously for human thioredoxin and ADF protein

Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetra-acetic acid; PBS, phosphate buffered saline; PMSF, phenylmethlysulphonyl fluoride.

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showed almost complete homology, suggesting that SASP is a membrane associated form of this thioredoxin group of proteins and may play a role in a wide number of biological activities that are depependent on dithiol reduction reactions.

MATERIALS AND METHODS

Cells Peritoneal cells were elicited from guinea pigs, Balb/c mice and Lewis rats with 2% (w./v.) starch in PBS and harvested 4 days later in PBS, 3mM EDTA, 0.5% bovine serum albumin. Normal human erythrocytes were purified on Ficoll hypaque, lysed in isotonic phosphate buffer pH 7.4 diluted x20 in water and the membranes washed repeatedly in the same buffer by centrifugation for 20min at 40,000g. Cell lines were from the Institute's own stocks and all cells were cultured in DMEM supplemented with 10% heat inactivated foetal calf serum, glutamine and penicillin/streptomycin at 37°C in 5% CO_2 humidified air.

Radiolabelling and detergent extraction After 2 washes in PBS, $5x10^5$ cells were resuspended in 30µl PBS containing 0.25mCi [^{14}C]-iodoacetamide (60mCi/mmol Amersham, Bucks. England) at 4°C for 10 min or at 37°C for up to 80min. Cold iodoacetamide was then added to a final concentration of 5mM, the cells washed x2 in PBS at 4°C and lysed by resuspending the pellet in 0.5% (v/v) Nonidet P-40 in PBS containing 5mM cold iodoacetamide, 2mM EDTA and 1mM phenylmethylsulphonyl fluoride (PMSF) for 30min at 4°C. Debris was removed by centrifugation at 11,000g for 15min.

Ultracentrifugation Those proteins solubilised by NP-40 were applied to a 5ml step sucrose gradient (5-30% sucrose in PBS) and centrifuged at 200,000g for 17h at 4°C on an SW60 Sorval rotor. 18 fractions were collected for analysis.

Electrophoresis SDS-PAGE was carried out on 5-20% and 10-20% gradient slab gels by the method of Laemmli [11]. Molecular weight markers in the range 200-14.3 kDa were purchased from Amersham (CFA-626). Low molecular weight standards in the range 16.9-6.2 kDa were supplied by Sigma (SDS-17). Cytochrome c (12.5 kDa) was used as an additional marker. Following impregnation of the gels with Amplify (Amersham) they were dried and kept in contact with Kodak XAR-5 X-ray film for 2-4 days at -70°C. Analytical isoelectric focusing (IEF) was performed over the ranges pH3.5-9.5 and 4-6.5 on pre-cast 1mM thick 5% polyacrylamide gels (2.2% ampholine w/v, Pharmacia, Uppsala, Sweden). Electrofocusing was carried out for 4h at 1W/cm (approximately 7500Vh). Gels were fixed and ampholines eluted in 10% TCA and proteins visualised by Coomassie blue staining and fluorography. Preparative focusing was carried out in a Biorad rotophor for 4h at 12W constant power, radiolabelled fractions collected and SASP further purified by chromatography on Sephadex G-75. Homogeneity was confirmed by reduced SDS-PAGE and purified samples of SASP transferred to "Immobilon" membranes overnight at 80mA constant current. The N-terminal amino-acid sequence was determined by the protein sequencing unit at Trinity College, Dublin using a gas phase protein sequencer.

RESULTS

When rat C6 glioma cells were radiolabelled with [^{14}C]-iodoacetamide at 37°C for 10min, proteins extracted in NP-40 and analysed by SDS-PAGE and fluorography, 3 major bands of 12.5kDa, 37kDa and 70 kDa were seen (fig 1). After 80min of labelling the number of bands had increased, so that the fluorograph resembled the Coomassie stained gel but the intensity of the 12.5 kDa band did not change. Labelling of dead cells or labelling of proteins after NP-40 extraction produced the pattern of many

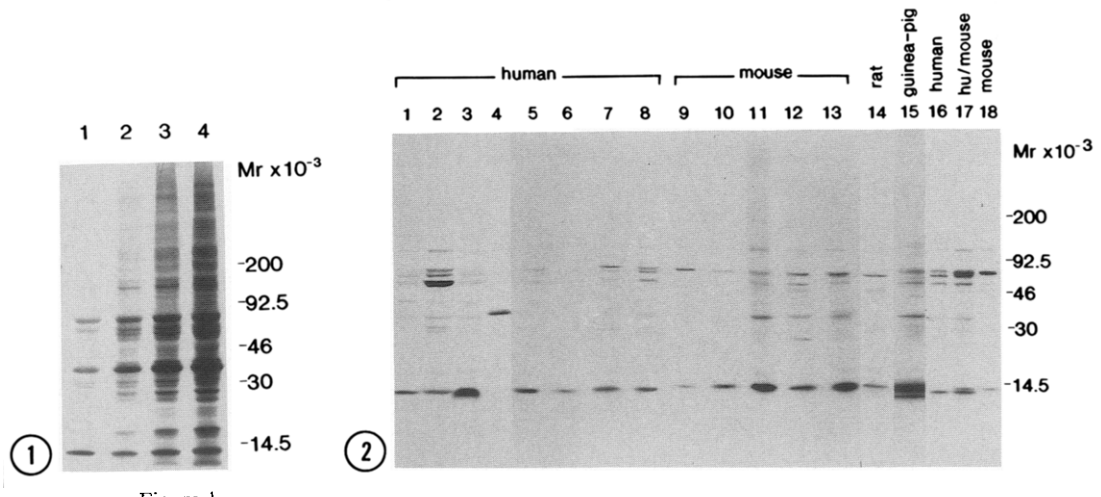

Figure 1

Rat glioma cells (5 x10^5 in 30µl PBS) were incubated with 0.25µCi (5 nmoles) [^{14}C]-iodoacetamide at 37°C for 10, 20, 40, and 80 min (lanes 1-4 respectively). NP-40 extracts of the cells were analysed by SDS-PAGE on a 5-20% gradient gel which was then fluorographed.

Figure 2

A variety of different cell types were labelled with [^{14}C]-iodoacetamide at 4°C for 10 min and the NP-40 extracts of each run on SDS-PAGE. The samples in each case were from: 1=IMR-32 neuroblastoma; 2=umbilical endothelium; 3=HeLa epithelial carcinoma; 4=erythrocyte ghosts; 5=U937 monocytoid; 6=HL-60 leukaemic myeloid; 7=THP-1 monocytoid; 8=normal fibroblast; 9=3T3 fibroblast; 10=peritoneal exudate; 11=NS1 B myeloma; 12=N_2A neuroblastoma; 13=C6 glioma; 14=peritoneal exudate; 15=peritoneal exudate; 16=fibroblast; 17=human fibroblast+ 3T3; 18=3T3.

labelled components seen in track 4. When a variety of cells from different species were labelled with iodoacetamide at 4°C it became apparent that the small 12.5kDa protein (SASP) was common to all cell types except for erythrocytes (fig. 2). A small but variable number of other sulphydryl containing proteins were also detected by this method but were less strongly labelled. In human cells the M_r of SASP was 1 kDa less than that extracted from mouse, rat and guinea-pig (fig. 2).

To determine whether SASP was a membrane associated protein, THP-1 cells were radiolabelled for 10 min at 4°C in the presence of 10mM azide and 5mM EDTA to minimise internalisation and then washed either in 0.2M acetate, 0.15M NaCl pH3.5 for 6min at 4°C or in PBS. Cells were pelleted, extracted in NP-40 and the radiolabel released into the supernatant and that remaining cell associated was analysed by SDS-PAGE and fluorography. The gels (fig.3) showed that SASP was readily stripped from the cells by the acid wash so that none remained in the NP-40 extract. In contrast, no SASP was removed by PBS and all the label was present in the NP-40 extract.

To determine if SASP was part of a larger protein complex, labelled extracts were applied to a 5-35% step sucrose gradient and samples from each fraction analysed by SDS-PAGE (fig. 4). The radiolabelled SASP band was not associated with a larger protein and was clearly distinguished in the upper fractions of the gradient along with other small proteins of similar molecular weight visible in the Coomassie stained gel.

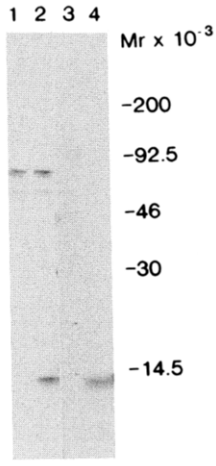

Figure 3
THP-1 cells in PBS containing 3mM EDTA and 10mM NaN_3 were labelled with [^{14}C]-iodoacetamide at 4°C. The cells were washed in the same buffer and placed in either fresh PBS or 150mM NaCl, 0.2M Na acetate buffer pH 3.5 for 6min at 4°C. The cells were then pelleted and proteins in the supernatant solutions precipitated with 5 volumes of acetone at -20°C for 1h in the presence of 100 µg bovine serum albumin as carrier. The cells were washed in cold PBS and lysed in NP-40. Protein in the acid treated and PBS washed cells (lanes 1 and 2 respectively) and the PBS and acid wash supernatants (lanes 3 and 4 respectively) was analysed by SDS-PAGE and fluorography.

Gel filtation on Sephadex G-75 in PBS, 0.5% NP-40 confirmed that SASP was a monomer, since it was separated from proteins larger than 15kDa (not shown) indicating that SASP was not part of a larger structure.

Samples of SASP extracted from rat C6 glial cells were subjected to isoelectric focusing and revealed a single distinct band with a pI of 4.8. When a detergent extract of $2x10^8$ human THP-1 monocytes was subjected to preparative IEF a single radioactive component was separated at a pI of 4.8 This SASP was further purified on

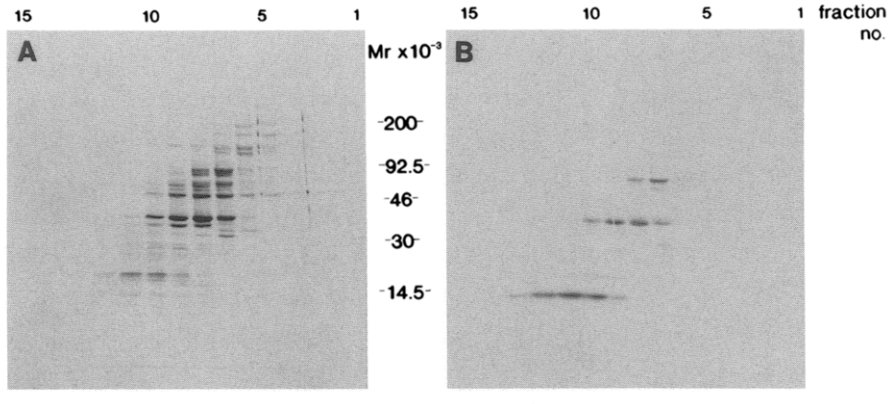

Figure 4
Rat C6 cells were radiolabelled with [^{14}C]-iodoacetamide at 4°C for 10min and solubilised with NP-40. The lysate was applied to a sucrose gradient, ultracentrifuged, fractionated and samples from each fraction subjected to SDS-PAGE.The resulting gel was stained with Coomassie blue (A) and fluorographed (B).

SASP:	V K Q I E S K T A F Q E ? L
Human thioredoxin:	M V K Q I E S K T A F Q E A L
Human ADF:	M V K Q I E S K T A F Q E A L

Figure 5
Comparison of the N-terminal amino acid sequences of SASP with human thioredoxin [14] and human ADF protein [10].

Sephadex G-75, eluting as one major band of radioactivity along with several minor labelled components and some low molecular weight material at the column V_t (not shown). The major component ran as a single band of M_r 11.5 kDa on a reduced SDS-PAGE gel stained with Coomassie blue. After electrophoretic transfer to "Immobilon" the N-terminal amino acid sequence of this component was determined and showed an unambiguous sequence up to residue 14 with a single amino acid terminus (fig. 5). This sequence was identical to the corresponding sequences deduced from cDNA for human thioredoxin and ADF protein, apart from the initial methionine residue.

DISCUSSION

Cells remained viable when monitored for 16h after they had been labelled for 10min with low concentrations of [^{14}C]-iodoacetamide. A large number of proteins were labelled by this procedure when non-viable or detergent solubilised cells were used or when viable cells were labelled for long periods of time. In contrast, very few bands were detected when cells were labelled for brief periods at 4°C, suggesting that these proteins were at or near the cell surface. The most prominent component was the low molecular weight SASP band, which was present in 2 forms, having an M_r of 12,500 in rat, mouse and guinea-pig cells and 11,500 in human. Both forms had a pI of 4.8. The SASP component was completely removed from viable cells when they were stripped for a brief period in acid buffer, an effective method for removal of membrane associated proteins [12, 13] showing that SASP was membrane associated.

SASP did not appear to be complexed with other membrane proteins since it behaved as a single component during gel filtration and centrifugation on sucrose gradients. It was present on all nucleated cells examined, including lymphocytes, fibroblasts, macrophages, epithelial, endothelial, neuronal and glial cells but absent from erythrocytes. Such widespread distribution suggested that SASP has a basic biological function, or functions not associated with specific specialised properties of this wide variety of cells. When SASP from human THP-1 monocytes was purified to homogeneity and its N-terminal amino acid sequence determined this was found to be almost identical to that deduced from a cDNA clone for human thioredoxin [14], which also has an almost identical M_r of 11,600 and a pI of 4.88. Thus, SASP appears to be a membrane associated form of the thioredoxin family of proteins. The thioredoxin sequence is itself identical to that deduced from the cDNA sequence of the ADF protein secreted by HTLV-1 transformed T cells except for 2 amino acid residues at positions 38 and 73 [10].

Thioredoxin is a ubiquitous dithiol-disulphide oxidoreductase that has a wide range of biological functions, including the ability to reduce and activate surface receptors [15]. The ADF protein has the ability to increase the number of IL-2/Tac receptors expressed by human YT-cells and transformed T cells by a mechanism that appears to involve a dithiol reduction [10, 16, 17]. Actively dividing cells appear to contain high levels of thioredoxin, while resting cells such as freshly isolated blood lymphocytes and monocytes do not [14]. Thus, pulse labelling of cells at 4°C provides a rapid and simple method for identifying membrane associated forms of the thioredoxin protein family. SASP appears to be a member of this family and may act as a growth factor by reducing and activating membrane receptors, such as the interleukin-2 Tac protein that have an important role in specific immune reactions.

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REFERENCES

1. Dereski, W. and Petty, H.R. (1985) Immunology 54, 397-399
Padgett,R.W., St.Johnston,R.D. and Gelbart,W.M. (1987) Nature 325, 81-84
2. Petty, H.R. (1985) Mol. Immunol. 22, 1001-1003
3. McAbee, D.D. and Grinnell, F. (1982) Biochem. J. 208, 473-478
4. Södeberg, B.O., Sjöberg, B.M., Sonnerstam,U. and Braüden, C.I. (1978) Proc. Natl. Acad. Sci.USA 75, 5827-30
5. Holmgren, A.,(1968) Eur. J. Biochem. 6, 475-84
6. Schürmann, P., Maeda, K., and Tsugita A. (1981) Eur. J. Biochem. 116, 37-45
7. Johnson, R.S., Mathews, W.R., Biemann, K. and Hopper S. (1988) J. Biol. Chem. 263, 9589-97
8. Rozell, B. and Hansson, H.A., Luthman, M. and Holmgren, A. (1985), Eur. J. Cell Biol. 38, 79-86
9. Stemme, S., Hanson, H.A., Holmgren, A. and Rozell, B. (1985) Brain Res. 359, 140-146
10. Tagaya, Y., Maeda, Y., Mitsui, A., Kondo, N., Matsui, H., Hamuro, J., Brown, N. Arai, K., Yokata, T., Wakasugi,H. and Yodoi, J. (1989) EMBO J., 8, 757-764
11. Laemmli,U.K. (1970) Nature 277, 680-685
12. Haigler, H.T., Maxfield, F.R., Willingham, M.C. & Pastan, I. (1980) J. Biol. Chem. 255, 1239-1241
13. Pelchen-Mathews, A., Armes, J.E. & Marsh, M. (1989) EMBO J. 8, 3641-3649
14. Wollman, E.E., d'Auriol, L., Rimsky, L., Shaw, A., Jaquot, J-P., Wingfield, P., Graber, P., Dessarps, F., Robin, P., Galibert, F., Bertologio, J., Fradelizi D., (1988) J. Biol. Chem. 263, 15506-15512
15. Grippo, J. F., Tienrungroj, W., Dahmer, M. J., Housley, P.R. and Pratt, W.P. (1983) J. Biol. Chem. 258, 13658-13664
16. Tagaya, Y., Taniguchi, Y., Naramura, M., Okada, M., Suzuki, N., Kanamori, H., Nikaido, T., Honjo, T. and Yodoi, J. (1987) Immunol. Lett. 15, 221-228
17. Tagaya, Y., Okada, M., Sugie, K., Kasahara, T., Kondo, N., Hamuro, J., Matsushima, K., Dinarello, C. A. and Yodoi, J., (1988) J. Immunol., 140, 2614-2620