J. Enzyme Inhibition, 1990, Vol. 3, pp. 311–316
Reprints available directly from the publisher
Photocopying permitted by license only

DIRECT EVIDENCE FOR THE CELL SURFACE LOCATION OF A PROTEASE-INHIBITOR COMPLEX ON INTACT LEUKAEMIA CELLS

F.S. STEVEN and M.M. GRIFFIN

Department of Biochemistry and Molecular Biology, School of Biological Sciences, University of Manchester, Manchester, M13 9PT, U.K.

(Received 4 October 1989)

The interaction of a protease with two fluorescent inhibitors has been studied using intact fixed leukaemia cells as the source of the membrane bound enzyme. Fresh rat leukaemia cells were disrupted and the cytosol collected; this extract was known to contain a protein inhibitor of guanidinobenzoatase (GB) associated with leukaemia cells. All the cytosolic proteins were derivatised with Texas red acid chloride. Leukaemia cells with latent GB failed to bind the Texas red inhibitor protein but did so after activation of GB. Competition experiments with 9-amino acridine (a fluorescent marker for the active site of GB) demonstrated that the Texas red — inhibitor protein could only bind to intact leukaemia cells when the active centre of GB was not already occupied by 9-amino acridine. This competition between these two fluorescent inhibitors demonstrated their specificity for GB. The use of intact leukaemia cells and the high molecular weight of the inhibitor protein precludes the possibility of any interaction between GB and inhibitor *within* the cells. It is concluded that GB and the GB — inhibitor complex of latent GB are located on the external surface of intact leukaemia cells.

KEY WORDS: Guanidinobenzoatase, Protease, Cell surface, Inhibitors, Enzyme-inhibitor complex.

INTRODUCTION

Our work is concerned with the protease guanidinobenzoatase[1,2] (GB) which is associated with tumour cells and cells capable of migration. GB is a trypsin-like enzyme with unusual substrate[1] and inhibition characteristics[3] which distinguishes it from most other trypsin-like proteases with the exception of the single chain form of tissue-type plasminogen activator.[4,5] Previous studies have shown that GB can be inhibited by tissue proteins extracted from homogenised cells[6] or from sectioned cells in frozen sections adhering to glass slides.[7] This inhibitor was shown to be displaced from the cell bound GB by formaldehyde treatment,[8] leaving the reactivated GB capable of binding the fluorescent probe 9-amino acridine (9-AA).

We have always considered this tumour cell associated protease to be on the external surface of the cells, in common with other such proteases.[9] In studies employing thin sections, the proof for such a belief is difficult to obtain in an unequivocal manner.

Leukaemia cells are transported by the blood and therefore do not require to have an active GB for cell migration. These cells possess a latent form of GB or "inhibited" GB. This inhibited GB can be taken advantage of in two ways:- (a) to define the location of GB on the cell, and (b) to provide protection for GB during adverse reaction conditions (see following paper). The recent demonstration that cell extracts can be used to prepare fluorescent protein inhibitors of GB[10] has now been extended

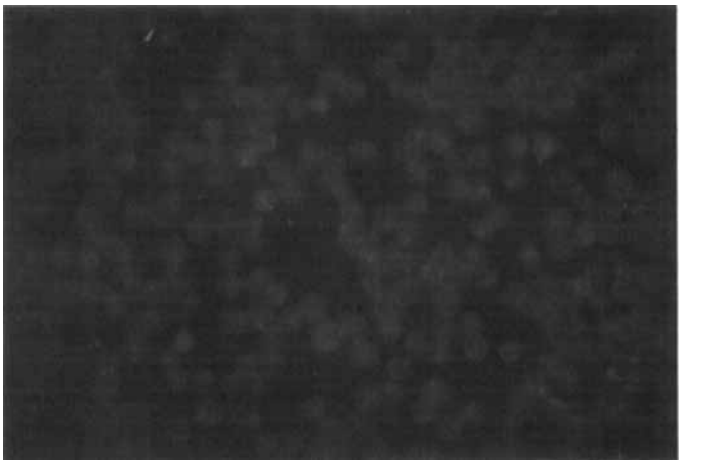

FIGURE 1 Fresh rat leukaemia cells treated with 9-AA. The cells show no surface binding of 9-AA and no yellow fluorescence. Filter cube [G], Magnification × 500. (See colour plate at back of issue).

to leukaemia cells. With this technique we can unequivocally define the site of GB to be on the external surface of intact leukaemia cells.

MATERIALS

9-amino acridine (9-AA) and Texas Red acid chloride (Sulphorhodamine 101 acid chloride) were purchased from Sigma Chemical Co. Ltd., St. Louis, Mo., USA. PD-10

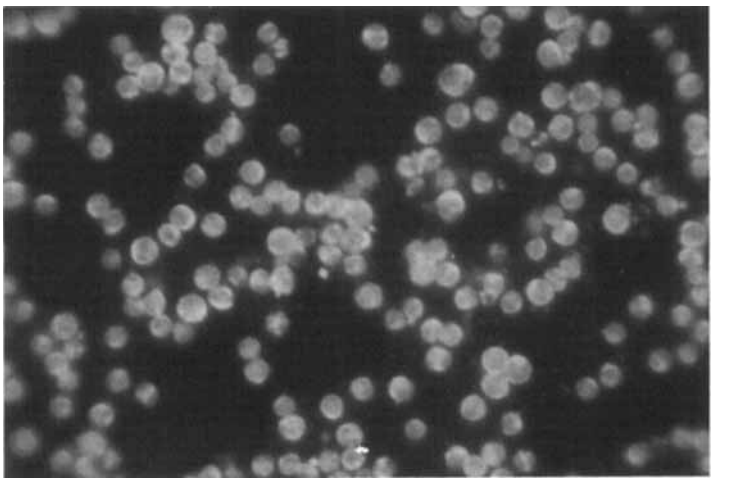

FIGURE 2 Rat leukaemia cells treated with formaldehyde prior to 9-AA. The cells now show cell surface binding of 9-AA and yellow fluorescence. This ability to bind 9-AA is due to the displacement of the inhibitor from the GB by the formaldehyde. Compare Figure 1 with Figure 2. Filter cube [G], Magnification × 500. (See colour plate at back of issue).

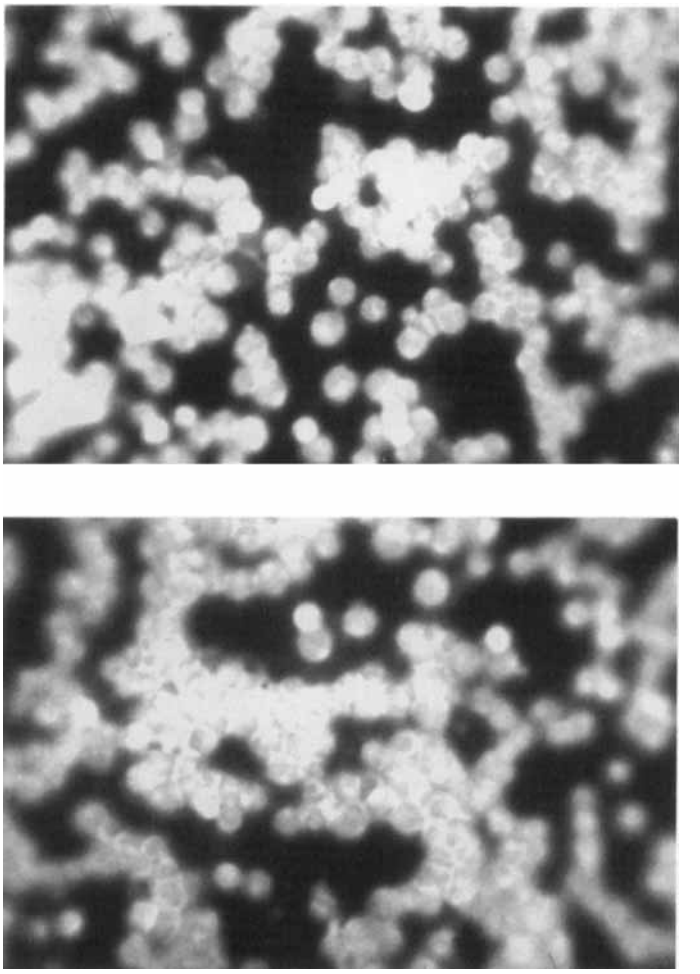

FIGURE 3,4 Rat leukaemia cells treated with formaldehyde and TR-I. The cells now bind the Texas red inhibitor of GB and exhibit red surface fluorescence. Fresh cells do not bind TR-I. Filter cube [N], Magnification × 500. (See colour plate at back of issue).

columns of Sephadex G-25M were obtained from Pharmacia AB, Uppsala, Sweden. Fresh rat leukaemia cells[11] were kindly provided by Dr. H. Jackson of the Department of Physiological Sciences, University of Manchester.

METHODS

Fresh rat leukaemia cells were placed on polylysine coated slides and made into cell smears. The smears were then allowed to dry at room temperature for 18 h. Formaldehyde treatment consisted of placing the dried smears in isotonic saline containing 10% stock formaldehyde (stock = 40%) i.e. having a final concentration of 4%.

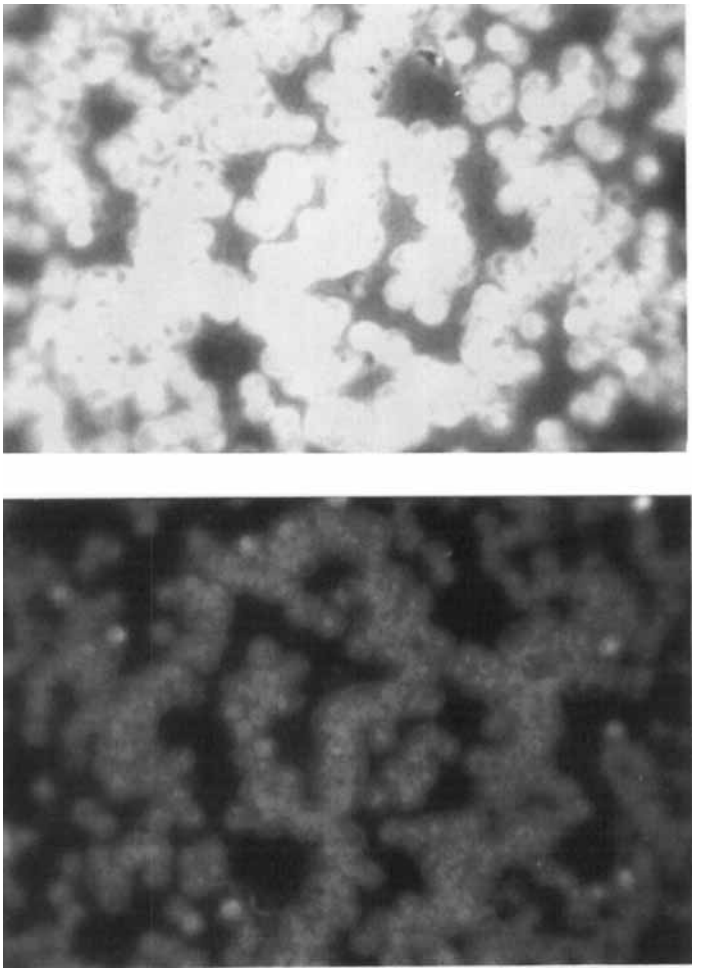

FIGURE 5,6 Rat leukaemia cells treated with formaldehyde and TR-I followed by 9-AA — Double labelling. The cells clearly bind TR-I (Figure 5) but have very little ability to bind 9-AA (Figure 6). This failure to bind 9-AA is due to the prior formation of GB-inhibitor complex with TR-I. Filter cube [N] for Figure 5; Filter cube [G] for Figure 6; Magnification × 500 for both Figures 5 and 6. (See colour plate at back of issue).

Fixation in formaldehyde was for 1 h followed by washing in isotonic saline containing $NaHCO_3$, 10^{-4}M, with a pH of 8.4 for 2 min.

Extraction of cytosolic proteins from the washed leukaemia cells was achieved by adding distilled water until the cells ruptured, releasing protein and DNA. The viscous DNA was removed by centrifugation from the soluble protein fraction; the latter being reacted with Texas Red acid chloride for 2 h; the protein fraction was then collected by passage through a PD-10 column.

The Texas Red labelled protein fraction contained the potential inhibitor (I) of the cell bound GB; for simplicity we refer to this as TR-I or the fraction containing the Texas Red labelled inhibitor.

Fluorescent labelling of the leukaemia cells

9-AA staining was carried out as previously described.[2] Briefly this requires the smears to be placed in a tank containing 9-AA (10^{-3}M) in isotonic saline for 2 min followed by washing in isotonic saline for 1 min.

Fluorescent labelling with TR-I

50 μl TR-I was placed upon the surface of the smear and left for 1 h at room temperature before the excess reagents were washed from the cells by placing in a tank of isotonic saline for 3 min.

Competition with 9-AA and TR-I

These experiments require two staining steps. When the effect of prior inhibition with TR-I was studied, the fluorescent labelling with TR-I was followed by direct 9-AA staining. In this case we knew that the GB-I complex was stable[8] and not displaced by subsequent treatment with 9-AA. When the effect of prior 9-AA labelling was studied, we added the TR-I to an equal volume of 9-AA solution in the second step, since 9-AA is bound *reversibly* in a concentration dependent manner.

Microscopic analysis

A Leitz Diaplan fluorescent microscope fitted with an Olympus OM-2_N camera with automatic exposure meter was employed. We used Kodak ASA 400, colour film throughout. For photography of yellow fluorescence, associated with the binding of 9-AA, we used filter cube [G] and barrier filter K490. The TR-I binding to cells was photographed using filter cube [N] and no additional barrier filter.

RESULTS AND DISCUSSION

The leukaemia cells initially lack the ability to bind 9-AA (Figure 1) and the cells have a greenish appearance similar to the background. After treatment with formaldehyde and subsequent exposure to 9-AA, the leukaemia cells now appear to fluoresce yellow, due to bound 9-AA (Figure 2). From previous studies,[6,8] these results can be interpreted as follows: formaldehyde treatment displaces an inhibitor (I) from the enzyme-inhibitor complex (GB-I) and subsequently the GB is able to bind 9-AA. This information does not define where in the leukaemia cells the GB-I is located, since 9-AA is small enough to enter the cell and react with it, either on the inside or the outside of the cell membrane. It is also known from previous studies that the cytosol of tumour cells contains soluble inhibitors (I) of GB. It would not be logical for both I and GB to be in contact with each other inside the cell membrane since this would not provide a functional enzyme system *in vivo*. The Texas Red inhibitor protein (TR-I) did not bind to leukaemia cells smeared on a glass slide (data not presented, since no fluorescence was observed). This indicated that neither specific nor non-specific binding of TR-I to GB took place on freshly smeared leukaemia cells.

When the leukaemia cells had first been treated with formaldehyde, the TR-I *did* bind and resulted in red fluorescence of the leukaemia cells (Figures 3,4). The

requirement of the formaldehyde pretreatment, for subsequent binding of TR-I, indicated that the GB-I on the original leukaemia cells prevented the binding of fresh TR-I to the leukaemia cells; exchange was not possible but binding of TR-I could take place once the pre-existing GB-I complex had been dissociated with formaldehyde. In Figures 3 and 4 the GB on the leukaemia cells is directly located by the red fluorescent TR-I obtained from the cytosol of similar leukaemia cells. This is due to the recognition of the cell bound GB by the TR-I to form GB-(TR-I).

We next considered the competition of 9-AA for the active centre of cell bound GB and the known ability of I to block this reaction.[6,8] The leukaemia cells were treated first with formaldehyde to activate the GB, then with TR-I, then with 9-AA. In this way, it could be determined whether the TR-I would block the arrival of 9-AA on the cell bound GB. It was observed that the presence of TR-I in the form of GB-(TR-I) blocked the subsequent arrival and binding of 9-AA (Figure 5). In this situation the leukaemia cells appear greenish with filter cube [G], i.e. lacking stacked 9-AA and similar to Figure 1. However these same leukaemia cells obviously contained TR-I as shown by their red fluorescence with filter cube [N] (Figure 6). Conversely formaldehyde treated leukaemia cells, treated directly with 9-AA appeared to fluoresce yellow with filter cube [G] (Figure 2), but showed no red fluorescence after competitive treatment with TR-I, when examined with filter cube [N] (data not shown).

The competition between 9-AA and TR-I for active GB confirms the data presented for studies with thin sections of tumour tissues,[10] however, the use of whole fixed cells in this study has one major advantage. It enables us to define on which side of the cell surface GB is located.

The cellular location of GB-(TR-I) complex must be on the *external surface* of the leukaemia cells since the TR-I (molecular weight[8] approximately 70,000) is far too large to pass through the cell membrane of the leukaemia cells. Thus the intracellular soluble inhibitor (I) would not normally be in contact with the cell surface GB unless the inhibitor was exported from the cell. This is entirely consistent with the use of such an inhibitor for the regulation of cell surface proteases[9] involved in cell migration.

Acknowledgements

FSS wishes to thank the Imperial Cancer Research Fund for the generous financial support without which this work would not have been carried out in Manchester.

References

1. Steven F.S. and Al-Ahmad, R.K. (1983) *Eur. J. Biochem,* **130,** 335.
2. Steven F.S., Griffin, M.M. and Al-Ahmad, R.K. (1985) *Eur. J. Biochem,* **149,** 35.
3. Steven, F.S., Griffin, M.M., Maier, H. and Altmannsberger, M. (1988) *J. Enz. Inhibit.,* **2,** 209.
4. Geiger, M. and Binder, B.R. (1987) *Biochim. Biophys. Acta,* **912,** 34.
5. Steven, F.S. and Griffin, M.M. *Biochem. Soc. Trans* (in press).
6. Steven, F.S., Griffin M.M., Freemont, A.J. and Johnson, J (1988) *J. Enz. Inhibit.,* **2,** 117.
7. Steven, F.S., Maier, H. and Arndt, J. *J. Enz. Inhibit.,* (in press).
8. Steven, F.S., Griffin, M.M., Wong, T.L.H. and Itzhaki, S. (1986) *J. Enz. Inhibit.,* **1,** 127.
9. Moscatelli, D. and Rifkin, D.B. (1988) *Biochim. Biophys. Acta,* **948,** 67.