The application of a novel biotinylated affinity label for the detection of a cathepsin B-like precursor produced by breast-tumour cells in culture

Breda M. CULLEN,* Isla M. HALLIDAY,† Gillian KAY,* John NELSON* and Brian WALKER*†

*Division of Biochemistry, School of Biology and Biochemistry, Queen's University, Belfast BT9 7BL, and †Department of Surgery, Institute of Clinical Science, Belfast BT12 6BJ, Northern Ireland, U.K.

In this report we demonstrate how the recently developed biotinylated affinity label biotinyl-Phe-Ala-diazomethane (Bio-Phe-Ala-CHN₂) [Cullen, McGinty, Walker, Nelson, Halliday, Bailie & Kay (1990) *Biochem. Soc. Trans.* **18**, 315–316; Walker, Cullen, Kay, Halliday, McGinty & Nelson (1992) *Biochem. J.* **283**, 449–453] can be used for the detection of a precursor form of a cathepsin B-like enzyme produced by breast-tumour cells in culture. Thus the cell lines MDA-MB-436, ZR-75-1 and T47-D produce a soluble protein that can be allowed to react with the biotinylated affinity label to yield an SDS-resistant complex; this can be revealed with a streptavidin/alkaline phosphatase label after PAGE and Western blotting. This protein (molecular mass 47 kDa) can also be detected by immunoblotting using sheep anti-(cathepsin B) antibodies in conjunction with a donkey anti-sheep IgG label. None of the cell lines studied produced any mature cathepsin B-like activity, as gauged by the lack of turnover of the fluorogenic substrate benzyloxycarbonyl-Arg-Arg-4-methylcoumarin-7-ylamide (Cbz-Arg-Arg-NH-Mec). However, treatment of medium samples with pepsin resulted in the generation of such activity. When the pepsin-catalysed activation step was analysed by SDS/PAGE, the protein of 47 kDa was completely converted into two species of very similar molecular masses of 30.5 kDa and 29 kDa. Both these proteins can incorporate the biotinylated probe and, in common with the 47 kDa species, they can be detected with the streptavidin/alkaline phosphatase label and immunoblotting. We propose that the 47 kDa form is the pepsin-activable proform of these lower-molecular-mass species. The release of the proform from the oestrogen-receptor (ER)-positive breast-tumour cell lines ZR-75-1 and T47-D is stimulated 5–10-fold when these cells are grown in medium containing epidermal growth factor (EGF) at a concentration of 10 ng/ml. In contrast, there is no modulation in the amount of proform released by the ER-negative cell line MDA-MB-436, over a range of EGF concentrations from 0 to 100 ng/ml.

INTRODUCTION

In the preceding paper (Walker *et al.*, 1992) we described the synthesis, kinetic characterization and application of the biotinylated active-site-directed inhibitor Bio-Phe-Ala-CHN₂, for the localization of cathepsin B activity in gels using Western-blot analysis in combination with a streptavidin/alkaline phosphatase detection system. We now wish to report on the utilization of this system for the characterization of the molecular forms of a cathepsin B-like enzyme secreted by the breast-tumour cells T-47D, ZR-75-1 and MDA-MB-436 in culture. In particular, we shall demonstrate how this probe can be used for the detection of the major secreted species of the enzyme, a pepsin-activable proform.

MATERIALS AND METHODS

Synthesis of affinity labels

The biotinylated affinity label biotinyl-Phe-Ala-diazomethane (Bio-Phe-Ala-CHN₂) was prepared by the procedure of Walker *et al.* (1992). The known diazomethane affinity labels, benzyloxycarbonyl (Cbz)-Phe-Ala-CHN₂ and Cbz-Phe-Tyr-(Obu[†])-CHN₂, were prepared by the general procedures of Shaw *et al.* (1983)

and had melting points in close agreement with the literature values (Shaw *et al.*, 1983; Kirschke *et al.*, 1988).

Materials for cell culture

All cell lines were obtained from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts., U.K.

Dulbecco's Modified Eagle's Medium (DMEM), foetal calf serum (FCS), penicillin and streptomycin were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K. NaHCO₃, glutamine and trypsin were supplied by Gibco, Paisley, Renfrewshire, Scotland, U.K. Sabaraud Dextrose broth, Nutrient broth and phosphate-buffered saline (PBS) tablets were supplied by Oxoid Ltd., Basingstoke, Hants., U.K. Tissue-culture flasks and 24-well cluster plates were from Costar, Northumbria Biologicals, Paisley, Renfrewshire, Scotland, U.K. Sterile disposable universal bottles were supplied by Sterilin, Stone, Staffordshire, U.K. Epidermal growth factor (EGF; murine, receptor grade) was supplied by Biogenesis, Bournemouth, Dorset, U.K., and was prepared in Earle's Balanced Salt solution (Gibco) containing 0.1% (w/v) BSA. Aliquots were stored at –20 °C.

Cell culture

MDA-MB-436 cells, which were originally established by Cailleau *et al.* (1978) from the pleural effusion of patients with breast cancer were cultured in L-15 medium supplemented with

Abbreviations used: Bio, biotinyl; Cbz, benzyloxycarbonyl; Boc, t-butoxycarbonyl; Mec, 4-methylcoumarin-7-yl; E-64, *trans*-epoxysuccinyl-L-leucylamino-(4-guanidino)butane; DMEM, Dulbecco's Modified Eagle's medium; FCS, foetal-calf serum; EGF, epidermal growth factor; PBS, phosphate-buffered saline; Obu[†], t-butyl ether; NBT, Nitro Blue Tetrazolium; BCIP, 5-bromo-4-chloroindol-3-yl phosphate; ER, oestrogen receptor; TGF α , transforming growth factor α ; BioP, biotinylated probe.

† To whom correspondence should be addressed.

10% (v/v) FCS, penicillin (100 i.u./ml) and streptomycin (100 µg/ml) and were passed weekly at a split ratio of 1:4.

T47D cells were cultured in DMEM containing 20 mM-Hepes, 7.5% (w/v) NaHCO₃, supplemented with 10% (v/v) FCS, penicillin (100 i.u./ml) and streptomycin (100 µg/ml). In common with the MDA-MB-436 cells, these cells were also originally obtained from pleural effusion of breast-cancer patients (Keydar *et al.*, 1976). The cells were passed weekly at a split ratio of 1:12.

ZR-75-1 cells, which were originally established from an ascitic effusion of a patient with metastatic mammary carcinoma (Engel *et al.*, 1978), were cultured in RPMI medium supplemented with 10% (v/v) FCS, penicillin (100 i.u./ml) and streptomycin (100 µg/ml).

All cells were grown at 37 °C in a Leec P3 dry incubator.

Steady-state assays

Before assaying for cathepsin B-like activity, cells were washed with phosphate-buffered saline (PBS; 0.05 M-sodium phosphate/0.1 M-NaCl, pH 7.4) and were then maintained for 48 h in the appropriate serum-free medium. This protocol was followed in order to remove residual serum proteins, especially proteinase inhibitors. After this 48 h incubation period the serum-free conditioned media from each of the tumour-cell lines was removed and briefly centrifuged at low speed to remove any cellular debris. Samples of the media were then assayed for cathepsin B-like activity with the fluorogenic substrate Cbz-Arg-Arg-NH-Mec (obtained from Bachem, Bubendorf, Switzerland), which is hydrolysed by cathepsin B but not cathepsins H or L (Barrett & Kirschke, 1981). Additionally, since kallikrein can also degrade this substrate (Mort & Leduc, 1984), control runs were always carried out in the presence of the active-site-directed inhibitor Cbz-Phe-Ala-CHN₂, which selectively inactivates cysteine, but not serine, enzymes (Green & Shaw, 1981).

Characterization of labelled proteinases

Labelling of proteinases. To samples (5 µl) of medium conditioned by the cell lines listed above was added 40 µl of 100 mM-phosphate buffer, pH 6.4, containing 2 mM-cysteine, 1 mM-EDTA and 0.1% (w/v) Brij 35. To this was added a solution (5 µl) of Bio-Phe-Ala-CHN₂ (1 mM) in methanol, and the solution was then incubated for 30 min at 37 °C. The reaction between enzyme and inhibitor was terminated by boiling the sample for 10 min with an equal volume of Laemmli treatment buffer.

Electrophoresis. Electrophoresis in the presence of SDS was performed by the method of Laemmli (1970) with 10% slab gels run at a constant current of 30 mA using the Mighty Small Apparatus (Hoefer Scientific). Labelled proteins were denatured by boiling for 10 min in 50 µl of Laemmli treatment buffer. Pre-stained molecular-mass markers (BRL) and biotinylated standards (Sigma) were included in each gel.

Western blotting

Western blotting was carried out by the method of Towbin *et al.* (1979). Transfer of protein from gel to nitrocellulose (0.2 µm pore size; Schleicher und Schüll, Dassel, Germany) was performed in 25 mM-glycine/192 mM-Tris/20% methanol using a Trans-Blot Cell (Bio-Rad). Running time was 18 h at 100 mA, and a constant temperature of 4 °C was maintained.

Detection of biotinylated proteins

After protein transfer, the blot was stained for biotinylated polypeptides by overlaying with streptavidin/alkaline phosphatase. The nitrocellulose sheet was first blocked by incubation for 30 min with a 5% solution of BSA (Sigma) containing 0.01% (v/v) merthiolate and 0.01% (v/v) anti-foam agent (Sigma). Streptavidin/alkaline phosphatase (Vector Laboratories), diluted

1:500 in blocking solution, was incubated with the nitrocellulose sheet for 5–24 h at room temperature with shaking. The blot was washed at least six times with PBS before the phosphatase activity was detected using Nitro Blue Tetrazolium (NBT) and 5-bromo-4-chloro-indol-3-yl phosphate (BCIP).

Immunoblotting

Samples of conditioned media that had not been exposed to biotinylated probe were subjected to electrophoresis and Western blotting as described above for the biotinylated proteins. Antibody probing of these Western blots was then carried out essentially as described by Maciewicz & Knight (1988). Sheep anti-human cathepsin B IgG (Serotec; used at 1:200 dilution) and donkey anti-sheep IgG conjugated to alkaline phosphatase (Serotec; used at 1:1000 dilution), were used as first and second antibodies respectively. Alkaline phosphatase activity was detected using NBT and BCIP.

RESULTS AND DISCUSSION

In the present study we examined media conditioned by a variety of breast-tumour-cell lines for the presence of cathepsin B-like activity. Thus, using a combination of methods involving fluorogenic-substrate assays, and our recently developed biotinylated affinity probe for cathepsin B-like proteinases (Cullen *et al.*, 1990; Walker *et al.*, 1992), we have partially characterized the cathepsin B-like enzymes produced by the oestrogen-receptor (ER)-negative cells, MDA-MB-436, and the ER-positive cells, T47D and ZR-75-1, in culture.

Quantification of cathepsin B-like activity in conditioned media: modulation by EGF

When samples of conditioned media from each of the cell lines were assayed for cathepsin B-like activity using the fluorogenic assay described above, no activity could be detected. This was irrespective of whether or not the medium was used 'neat' or concentrated 20-fold by ultrafiltration (10 kDa cut-off membrane at 4 °C).

However, since pepsin-activatable precursor forms of cathepsin B-like proteinases have been detected in ascitic fluid of patients with ovarian cancer (Mort *et al.*, 1980, 1981, 1983) and in ascites cells in culture (Keppler *et al.*, 1988), we decided to apply the pepsin-activation protocols essentially as described by these workers, in the hope of activating any latent cathepsin B-like activity that might be present in the conditioned media from the breast-cancer cells. Fortunately, treatment of samples of these media with pepsin (Sigma, final concn. 20 µg/ml), in 0.1 M-glycine/HCl buffer, pH 3.0, at 37 °C, resulted in the formation of readily detectable amounts of cathepsin B-like activity, as measured by the turnover of Cbz-Arg-Arg-NH-Mec. The hydrolysis of this substrate could always be blocked by the diazomethane inhibitor Cbz-Phe-Ala-CHN₂, a known inactivator of cysteine proteinases (Green & Shaw, 1981).

Fig. 1 shows the time course for the generation of such activity from medium conditioned by MDA-MB-436 cells after pepsin treatment (similar activation profiles were obtained for the two ER-positive cell lines; results not shown). It can be seen from this Figure that maximum cathepsin B-like activity is generated after exposure to pepsin for approx. 60 min. In the absence of pepsin, no activation of latent enzyme was observed in samples of medium from any of the cell lines studied. This lack of activation in acidic medium alone sets this latent species apart from the latent, but acid-activatable, cathepsin L-like species secreted from Kirsten-Virus-transformed NIH 3T3 mouse fibroblasts (Gal & Gottesman, 1986a,b; Troen *et al.*, 1987). However, the requirement for proteolytic involvement in the activation is

Fig. 1. Time course for the pepsin-catalysed activation of latent cathepsin B-like activity in the medium conditioned by the breast-tumour cells (MDA-MB-436) in culture

Activation of medium samples conditioned by the tumour cells was carried out as described in the Results and discussion section. The traces show the generation of cathepsin B-like activity (as measured by the turnover of the fluorogenic substrate Cbz-Arg-Arg-NH-Mec) in the presence (■) or absence (□) of pepsin.

Table 1. Amounts of procathepsin B-like material released by the breast-tumour cells, MDA-MB-436, ZR-75-1 and T47-D, in culture

Values given (means ± S.E.M. for four determinations) are for the amount of mature cathepsin B-like activity (as measured by the turnover of Cbz-Arg-Arg-NH-Mec) generated by treatment of medium samples with pepsin as outlined in the Results and discussion section.

Cell line and medium treatment	Amount of cathepsin B-like activity (ng/10 ⁶ cells)
MDA-MB-436 (L-15 medium, no EGF)	360 ± 118
MDA-MB-436 (L-15 medium, 10 ng of EGF)	377 ± 42
MDA-MB-436 (L-15 medium, 100 ng of EGF)	403 ± 32
T-47D (DMEM medium, no EGF)	16 ± 2
T-47D (DMEM medium, 10 ng EGF)	84 ± 3
ZR-75-1 (RPMI medium, no EGF)	1 ± 0.5
ZR-75-1 (RPMI medium, 10 ng EGF)	9 ± 2

entirely in keeping with the properties described for the latent cathepsin B-like species detected in ascitic fluid from women with ovarian carcinoma (Mort *et al.*, 1980, 1981, 1983; Keppler *et al.*, 1988). Table 1 lists the levels of cathepsin B-like activity detected in the media conditioned by the three breast-tumour cell lines used in the present study. The activity is expressed as the amount (in ng) of cathepsin B-like material [determined by active-site titration with E-64 according to the protocols of Barrett & Kirschke (1981)] produced per 10⁶ cells and which is released into the medium as a latent/proform that can be activated by pepsin. A number of features are immediately apparent from this Table. Firstly, upon pepsin treatment, clear differences are revealed between the levels of activatable latent cathepsin B-like activity present in medium conditioned by the ER-negative and ER-positive cells. Thus medium from the MDA cells contains

Fig. 2. Western-blot analysis of samples of conditioned medium from MDA-MB-436 cells probed with Bio-Phe-Ala-CHN₂ or sheep anti-(human cathepsin B) antibodies

Samples of medium conditioned by the breast-tumour cells were split in half, and one portion was treated with pepsin (20 µg/ml for 1 h at 37 °C) as described in the Results and discussion section. Portions of both samples were then treated with the biotinylated affinity label (see the Materials and methods section) and the remaining portions were utilized for immunoblotting. All sets of samples were then subjected to electrophoresis on a 10%-(w/v)-polyacrylamide gel run under reducing and denaturing conditions. Electrophoretic transfer of the proteins on to nitrocellulose was then carried out as outlined in the Materials and methods section. Lanes A and B represent the immunoblots of the medium before and after pepsin treatment respectively. Lanes C and D are Western blots of the samples of medium that were subjected to incubation with the biotinylated affinity label before and after pepsin activation respectively.

substantially higher levels of latent cathepsin B-like material than does that from the ER-positive cells. For example, the MDA cells secrete 360 ± 80 ng of pepsin-activatable latent cathepsin B-like material per 10⁶ cells in comparison with a range of ~ 1–16 ng from equivalent numbers of the ER-positive cells.

The difference between the two classes of tumour cells is further highlighted by their release/secretion of this latent activity when they are cultured in medium containing EGF. Thus both the T-47D and the ZR-75-1 cells secrete between 5- and 9-fold more latent enzyme upon addition of EGF to the medium (final concn. of 10 ng/ml), whereas with the ER-negative cells, the secretion/release of this latent species does not appear to be modulated by the addition of EGF over a range of concentrations from 10 to 100 ng/ml.

A possible explanation for this unresponsiveness may be found in the observation that this cell line secretes the EGF homologue transforming growth factor α (TGFα) into the culture medium (Quaroni *et al.*, 1979; Imanishi *et al.*, 1989) and thus may be under autocrine control by TGFα/EGF-like molecules. The production of the latent cathepsin B-like material could also be subject to the same autocrine loop, and consequently, addition of exogenous EGF may not be able to bring about a further increase in the secretion/release of this precursor molecule.

Characterization of the molecular forms of the cathepsin B-like enzyme produced by breast-tumour cells in culture

Samples of the conditioned media from the three breast-tumour-cell lines were also examined for the presence of cathepsin B-like activity using the biotinylated affinity label Bio-Phe-Ala-CHN₂, which we have recently synthesized and shown to be useful as a probe for the detection of active human and bovine cathepsin B (Cullen *et al.*, 1990; Walker *et al.*, 1992). Fig. 2 shows the results of the Western-blot analysis carried out on samples of the conditioned medium from the MDA-MB-436 cells that were incubated with the biotinylated affinity label, before and after pepsin treatment, and after the medium sample

had been subjected to SDS/PAGE on a 10% gel, run under reducing conditions. Also shown for comparison are the immunoblots of the medium before and after pepsin treatment, carried out using sheep anti-(human cathepsin B) antibodies.

Lanes C and D show representative results of the probing of the conditioned media from the MDA-MB-436 cells using the biotinylated inhibitor before and after pepsin treatment respectively. Thus incubation of a sample of the conditioned medium with the biotinylated probe reveals the presence of a single, but diffuse, protein band of apparent molecular mass 47 kDa before pepsin treatment; this species is completely converted by the action of the proteinase into two components of very similar molecular masses (30.5 and 29 kDa).

These two low-molecular-mass species could arise from any one of three general pathways. Firstly, they could be the result of the cleavage of a single substrate molecule at two different loci. Secondly, pepsin could cleave this putative single precursor molecule at only one position to yield the 30.5 kDa species, which could be further proteolysed (either by pepsin or by autolysis) to yield the 29 kDa form. Thirdly, particularly in view of the diffuse nature of the band at 47 kDa, they could have arisen from a single pepsin-catalysed cleavage in each of two putative high-molecular-mass isoenzyme precursors that are not resolvable under the electrophoretic conditions used in the present study. The precedence for the existence of such isomorphous precursor forms of mammalian cathepsin B is well documented [see, e.g., Takahashi *et al.*, (1984a,b, 1986) and Hanewinkel *et al.* (1987)]. However, these reports can only be considered at best as providing circumstantial evidence in support of the third possibility, and further experiments are required in order to definitively establish which of the three possibilities is correct.

Although we expected to detect the presence of the mature form of the proteinase with this affinity label, the incorporation of this reagent into the latent proform was entirely unexpected, since we had assumed that the medium contained no catalytically competent species, as gauged by the lack of turnover of the fluorogenic substrate Cbz-Arg-Arg-NHMeC before 'activation'. This precursor form can also be detected by the biotinylated probe (BioP) in the conditioned media of both of the ER-positive tumour-cell lines (results not shown).

The covalent modification of the proforms by BioP is completely blockable by pretreatment of a sample of the medium with either iodoacetamide (10 mM for 30 min at 37 °C) or Cbz-Phe-Ala-CHN₂ (50 µM, for 30 min at 37 °C), a known active-site-directed affinity label for cathepsins B and L (Watanabe *et al.*, 1979; Green & Shaw, 1981; results not shown). This ability to block the incorporation of BioP into the 47 kDa species is totally in keeping with our observations on the active-site-labelling of mature bovine and human lysosomal cathepsin B reported in the accompanying paper (Walker *et al.*, 1992) and strongly suggests that BioP is functioning as a true active-site-directed affinity label of the proform.

The inability of the proform to 'turn over' the substrate but yet react with the affinity label would appear at first sight to represent something of a dichotomy. However, the recent report on the incorporation of an ¹²⁵I-radiolabelled epoxide derivative *N*-(2*S*,3*S*)-3-*trans*-ethoxycarbonyloxirane-2-carbonyl-L-tyrosine benzyl ester (an analogue of E-64), into the 'catalytically inactive' prepro form of papain (Vernett *et al.*, 1991), corroborates our observations and would suggest that the proforms of the papain superfamily of proteinases do possess some inherent chemical reactivity towards certain active-site-directed affinity labels.

Probing of the conditioned media from each of the three breast-tumour-cell lines with anti-(cathepsin B) antibodies gave

results exactly comparable with those obtained with BioP. Thus lanes A and B in Fig. 2 show the results of the immunoblotting studies carried out on a sample of the medium conditioned by the MDA-MB-436 cells before and after pepsin treatment respectively. It can be seen that the antibodies also label a single, but diffuse, species of apparent molecular mass 47 kDa before pepsin treatment and subsequently recognize two components of a very similar molecular mass (30.5 and 29 kDa) after the sample has been exposed to pepsin.

The absolute symmetry between the results of the labelling experiments carried out with the biotinylated probe and the anti-(cathepsin B) antibodies, in combination with the lack of 'autocatalysis' of the proform in acidic medium alone, lends further support to our thesis that we are detecting a proform of cathepsin B and not cathepsin L.

The detection and characterization, using antibodies raised against active human cathepsin B, of latent forms of the enzyme secreted by various tumour cells in culture, has been reported previously by other workers. For example, a latent and pepsin-activatable enzyme of 38 kDa from medium conditioned by primary cultures of colorectal tumours has been partially characterized (Maciewicz *et al.*, 1989). Similarly, malignant ascites cells in culture are known to produce a 40 kDa proform of the enzyme which can be converted into a 33 kDa species by pepsin treatment (Mort *et al.*, 1983). Of even more direct relevance to this present study is the report by Mort & Recklies (1986) of the detection of a 40 kDa latent cathepsin B-like species in the medium conditioned by primary cultures of human mammary adenocarcinomas. This latent form can also be converted by pepsin into an active species capable of hydrolysing fluorogenic substrates containing double basic residues. Finally, the presence of a pepsin-activatable 45–47 kDa latent cathepsin B-like species detected in the ascitic fluid of a patient with ovarian carcinoma (Keppler *et al.*, 1988), most closely resembles the precursor molecule discussed in the present study, at least in terms of apparent molecular mass.

Generous financial support from the Cancer Research Campaign and the funding of a studentship by Action Cancer (to B.M.C.) are gratefully acknowledged.

REFERENCES

- Barrett, A. J. & Kirschke, H. (1981) *Methods Enzymol.* **80**, 535–561
- Cailleau, R., Olive, M. & Cruciger, J. (1978) *In Vitro* **14**, 911–915
- Cullen, B. M., McGinty, A., Walker, B., Nelson, J., Halliday, I. M., Bailie, J. R. & Kay, G. (1990) *Biochem. Soc. Trans.* **18**, 315–316
- Engel, L. W., Young, N. A., Tralka, T. S., Lippman, M. E., O'Brien, S. J. & Joyce, M. J. (1978) *Cancer Res.* **38**, 3352–3364
- Gal, S. & Gottesman, M. M. (1986a) *J. Biol. Chem.* **261**, 1760–1765
- Gal, S. & Gottesman, M. M. (1986b) *Biochem. Biophys. Res. Commun.* **139**, 156–162
- Green, G. D. J. & Shaw, E. (1981) *J. Biol. Chem.* **256**, 1923–1928
- Hanewinkel, H., Glossl, J. & Kresse, H. (1987) *J. Biol. Chem.* **262**, 12351–12355
- Imanishi, K., Yamaguchi, K., Suzuki, M., Honda, S., Yanahara, N. & Abe, K. (1989) *Br. J. Cancer* **59**, 761–777
- Keppler, D., Pagano, M., Dalet-Fumeron, V. & Engler, R. (1988) *Biol. Chem. Hoppe-Seyler* **369** (Suppl.), 185–190
- Keydar, I., Chen, L., Karby, S., Weiss, F. R., Delares, J., Radu, M., Chaitcik, S. & Brenner, H. (1976) *Eur. J. Cancer* **15**, 659–670
- Kirschke, H., Wikstrom, P. & Shaw, E. (1988) *FEBS Lett.* **228**, 128–130
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Maciewicz, R. A. & Knight, P. J. (1988) *Anal. Biochem.* **175**, 85–90
- Maciewicz, R. A., Wardle, R. J., Etherington, D. J. & Paraskeva, C. (1989) *Int. J. Cancer* **43**, 478–486
- Mort, J. S. & Leduc, M. C. (1984) *Clin. Chim. Acta* **140**, 173–182
- Mort, J. S. & Recklies, A. D. (1986) *Biochem. J.* **233**, 57–63
- Mort, J. S., Recklies, A. D. & Poole, A. R. (1980) *Biochim. Biophys. Acta* **614**, 134–143

- Mort, J. S., Leduc, M. C. & Recklies, A. D. (1981) *Biochim. Biophys. Acta* **662**, 173–180
- Mort, J. S., Leduc, M. C. & Recklies, A. D. (1983) *Biochim. Biophys. Acta* **755**, 369–375
- Quaroni, A., Wands, J., Trelsted, R. & Isselbacher, K. (1979) *J. Cell Biol.* **80**, 248–265
- Shaw, E., Wikstrom, P. & Ruscica, J. (1983) *Arch. Biochem. Biophys.* **222**, 424–429
- Takahashi, T., Schmidt, P. G. & Tang, J. (1984a) *J. Biol. Chem.* **259**, 6059–6062
- Takahashi, T., Dehdarani, A., Schmidt, P. G. & Tang, J. (1984b) *J. Biol. Chem.* **259**, 9874–9882
- Takahashi, T., Yonezawa, S., Dehdarani, A. H. & Tang, J. (1986) *J. Biol. Chem.* **261**, 9368–9374
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Troen, B. R., Gal, S. & Gottesman, M. M. (1987) *Biochem. J.* **246**, 731–735
- Vernett, T., Khouri, H. E., Tessier, D. C., Laflamme, P. Storer, A. C. & Thomas, D. Y. (1991) *J. Cell. Biochem. Suppl.* **15G**, CH 319
- Walker, B., Cullen, B. M., Kay, G., Halliday, I. M., McGinty, A. & Nelson, J. (1992) *Biochem. J.* **283**, 449–453
- Wantanbe, H., Green, G. D. J. & Shaw, E. (1979) *Biochem. Biophys. Res. Commun.* **89**, 1354–1360

Received 31 July 1991/9 October 1991; accepted 9 October 1991