Regulation of natural killer cell-mediated cytotoxicity by serine/threonine phosphatases: identification of a calyculin A-sensitive serine/threonine kinase

Anil BAJPAI* and Zacharie BRAHMI*†‡

Departments of *Medicine and †Microbiology and Immunology, Indiana University School of Medicine, 702 Barnhill Drive, Riley 0615, Indianapolis, IN 46202, U.S.A.

We have recently reported that Ser/Thr phosphatases play a key role in regulating natural killer (NK) cell lytic activity and that calyculin A and okadaic acid affect this activity differently [Bajpai and Brahmi (1994) *J. Biol. Chem.* **269**, 18864–18869]. Here, we investigate a mechanism that might account for this differential action of calyculin A and okadaic acid on NK cells. Calyculin A specifically inhibited the lytic activity of YT-INDY, an NK-like cell line, and hyperphosphorylated 60 and 78 kDa proteins. The kinetics of appearance of these two proteins was correlated with the loss of lytic activity. In contrast, okadaic acid did not significantly affect either of these activities. The 78 kDa protein is localized in the cytosolic compartment whereas the

60 kDa protein is distributed equally between the membrane and the cytosolic fractions. Both proteins display a kinase activity and are phosphorylated mainly at serine and threonine residues but not at tyrosine residues. The activation of these kinases is specific to calyculin A treatment; it is independent of protein kinase C, protein kinase A, Ca^{2+} , phosphotyrosine phosphatase and protein synthesis *de novo*. In conclusion, we have demonstrated that calyculin A, but not okadaic acid, hyperphosphorylates two proteins with Ser/Thr kinase activity, thus explaining the differential regulation of NK cells by these two Ser/Thr phosphatase inhibitors.

INTRODUCTION

Natural killer (NK) cells spontaneously lyse a variety of tumour cells either by direct cell-to-cell contact (cell-mediated cytotoxicity; CMC) or via anti-target cell antibodies (antibody-dependent cellular toxicity) without prior sensitization [1]. Thus in contrast with cytolytic T-lymphocytes (CTLs), they provide the first line of defence against certain forms of cancer [1]. NK cell-mediated lysis involves the recognition and binding of as yet unidentified motifs on target cells and the subsequent Ca^{2+} -dependent release of a number of lytic molecules that ultimately induce target cell lysis [2–4].

The exact biochemical events of NK-CMC are not well understood, although it is known that after activation NK cells undergo extensive phosphorylation and dephosphorylation of cellular proteins [5]. Recent studies have implicated several tyrosine kinases as well as CD45, a receptor-like phosphotyrosine phosphatase (PTPase) in the regulation of the lytic activity of NK cells. Cell-surface-receptor activation induces the transduction of intracellular signals through extensive Ser/Thr phosphorylation [5,6]. The interplay between various Ser/Thr kinases and phosphatases in the regulation of the activation of cytolytic lymphocytes after binding to tumour targets is still unresolved. The regulation of CD45-associated PTPase activity by Ser/Thr phosphorylation is well documented but the exact effect of Ser/Thr phosphorylation on CD45 activity is still unknown. Yamada et al. [7] reported that increased phosphorylation of CD45 by protein kinase C (PKC) down-regulates CD45 PTPase activity whereas treatment of CD45 with

PKC *in vitro* did not significantly affect its PTPase activity [8]. Valentine et al. [9] suggested that interleukin 2 (IL-2)-induced serine phosphorylation of CD45 has no effect on the cell-surface expression of this PTPase or on its activity. In contrast, Ostergaard and Trowbridge [10] reported a direct link between phosphorylation at serine residues and CD45 activity.

We have recently shown that Ser/Thr phosphatases and PTPases were equally important in NK-CMC and that calyculin A and okadaic acid affected NK-CMC differently [11]. Here we report that in an NK-like cell line, YT-INDY, calyculin A induces a 60 kDa and a 78 kDa protein with Ser/Thr phosphotransferase (kinase) activity and that their expression is correlated with the loss of NK lytic function. In contrast, okadaic acid, which does not significantly affect NK-CMC, has no effect on the activation of these kinases. These proteins are phosphorylated only at Ser/Thr residues with no detectable phosphotyrosine. We propose that calyculin A may be regulating NK-CMC through these two Ser/Thr kinases.

EXPERIMENTAL

Cell line and inhibitors

YT-INDY, an NK-like subline cloned from the original NK-like cell line YT, was used throughout this study [12,13]. YT-INDY was routinely maintained in complete medium consisting of RPMI 1640 containing 2 mM L-glutamine, 10% (v/v) cosmic calf serum (Hyclone Laboratories, Logan, UT, U.S.A.),

Abbreviations used: BIS, bisindolylmaleimide I, hydrochloride; CMC, cell-mediated cytotoxicity; CTL, cytolytic T-lymphocyte; IEF, isoelectric focusing; IL-2, interleukin 2; MAP, mitogen-activated protein; NK, natural killer; PKA, protein kinase A; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP1, PP2A, protein phosphatase 1 and 2A; PTPase, phosphotyrosine phosphatase.

‡ To whom correspondence should be addressed at Department of Medicine, Riley Hospital, Room no. 0615, 702 Barnhill Drive, Indianapolis, IN 46202, U.S.A.

100 $\mu\text{g/ml}$ streptomycin and 100 i.u./ml penicillin (Sigma Chemical Co., St. Louis, MO, U.S.A.). Cell viability was determined by Trypan Blue dye exclusion. Calyculin A and okadaic acid were purchased from Kamiya Biochemical Co. (Thousand Oaks, CA, U.S.A.) and were dissolved in DMSO. The DMSO concentration was always less than 0.1 % (v/v) and at this concentration had no effect on cell viability or lytic function.

Cytotoxicity assay

Cytotoxicity assays were done as described previously [14]. In brief, ^{51}Cr -labelled target cells (0.1 ml) were mixed with YT-INDY (0.1 ml) in 96-well round-bottomed microtitre plates (Costar, Cambridge, MA, U.S.A.) to yield a lymphocyte-to-target cell ratio of 20:1. The plates were incubated at 37 °C for 4 h, centrifuged for 5 min at 400 g and room temperature and the supernatant from each well was collected with a Skatron supernatant collection system (Skatron, Sterling, VA, U.S.A.). Spontaneous release was determined by measuring radioactivity in the wells containing 100 μl of target cells and 100 μl of medium. The maximum c.p.m. was determined by counting 100 μl of labelled target cells. The percentage lysis was calculated as $100 \times [(\text{test c.p.m.} - \text{spontaneous c.p.m.}) / (\text{maximum c.p.m.} - \text{spontaneous c.p.m.})]$. Values in triplicate for each group were averaged.

Western blotting

Cells were treated with calyculin A or okadaic acid as described in the figure legends. After treatment the cells were washed twice with chilled PBS and transferred to a lysis buffer [50 mM Tris (pH 7.4)/100 mM NaCl/10 mM EDTA/1 % NP40/0.1 % SDS/1 mM sodium orthovanadate/1 mM PMSF/100 $\mu\text{g/ml}$ each of leupeptin, aprotinin and pepstatin] for 30 min at 4 °C. Cell lysates were centrifuged at 14000 g and 4 °C for 10 min in a microcentrifuge, mixed with an equal volume of Laemmli sample buffer and boiled for 2 min. Proteins were separated on 10 % (w/v) gel by SDS/PAGE [15] and transferred to Immobilon-P membranes (Millipore, Bedford, MA, U.S.A.) for 1 h at 25 V with a Mini Trans-Blot cell (Bio-Rad, Melville, NY, U.S.A.).

Kinase renaturation assay

The renaturation and measurement of enzymic activity of blotted protein kinases were done as described earlier [16]. In brief, the blotted proteins were denatured for 1 h at room temperature in 7 M guanidine hydrochloride. The blots were briefly washed in Tris-buffered saline and incubated for 16–18 h in renaturation buffer. After incubation the blots were blocked for 1 h in 30 mM Tris/HCl (pH 7.4) containing 5 % BSA, and the blocking solution was discarded. The kinase activity was measured for 30 min at room temperature, in a buffer containing 30 mM Tris/HCl, pH 7.4, 10 mM MgCl_2 , 2 mM MnCl_2 and 50 $\mu\text{Ci/ml}$ [$\gamma\text{-}^{32}\text{P}$]ATP (3000–6000 Ci/mmol). The filters were washed as described [16].

Phosphoamino acid analysis

The bands of interest were excised from the blots and digested in 6 M HCl for 1 h at 110 °C. After incubation the lysate was dried in vacuum and the pellet washed twice with water. The samples were analysed on cellulose plates (Eastman Kodak, Rochester, NY, U.S.A.) at 500 V for 70–80 min at room temperature. Internal standards of phosphoamino acids were included in the digestion and were detected by ninhydrin spray. Autoradiography was done on X-Omat/AR X-ray films.

RESULTS

Effect of calyculin A and okadaic acid on YT-INDY CMC

We have previously shown that calyculin A and okadaic acid, two Ser/Thr phosphatase inhibitors, affected NK-CMC differently [11]. Calyculin A inhibited both CTL and NK cell lytic activity whereas okadaic acid inhibited only CTL without significantly affecting NK cells. To evaluate further the role of Ser/Thr phosphatases in NK-CMC, we used an IL-2-independent NK-like cell line, YT-INDY, that can lyse several B-cell and a few T-cell lines [12,13]. YT-INDY cells constitute a homogeneous population of cloned effector cells that are required to study the mechanism of action of these inhibitors. The effect of calyculin A and okadaic acid on YT-INDY-CMC (Figure 1) was similar to that observed with fresh NK cells [11]. Calyculin A inhibited YT-INDY-CMC in a dose-dependent manner with almost complete inhibition at approx. 20 nM whereas okadaic acid had essentially no effect at up to 1 μM . Incubation of YT-INDY with 2.5, 5, 10 and 20 nM calyculin A for 30 min at 37 °C resulted in 0.8 %, 13 %, 67 % and more than 98 % inhibition respectively (Figure 1). Our studies showed that approx. 8 nM calyculin A was needed for 50 % inhibition of YT-INDY-CMC. Throughout the rest of the study we, therefore, routinely pre-incubated YT-INDY with 20 nM calyculin A and 1 μM okadaic acid for 30 min at 37 °C.

Effect of calyculin A and okadaic acid on cellular kinases

The regulation of kinases and phosphatases plays a key role in a number of lymphocyte functions [5–7]. To determine the effect of calyculin A and okadaic acid on cellular kinases, we treated YT-INDY cells with these two inhibitors, separated the proteins by SDS/PAGE and then tested for in-gel kinase activity [16]. This assay has regularly been used to determine the activity of mitogen-activated protein (MAP) and other kinases [17–20]. We observed several protein bands with kinase activity in calyculin A-treated and okadaic acid-treated cells (Figure 2). In particular there were two protein bands at 78 and 60 kDa with strong kinase activity

Figure 1 Kinetics of calyculin A- and okadaic acid-mediated inhibition of YT-INDY-CMC

YT-INDY cells were treated with calyculin A or okadaic acid for 30 min at 37 °C, washed and assayed in a standard 4 h ^{51}Cr release assay. Raji, an EBV transformed cell line, was used as target cells.

Figure 2 Effect of calyculin A and okadaic acid on the kinase activities of YT-INDY cells

YT-INDY cells were incubated alone (CON), with 20 nM calyculin A (CAL), with 1 μ M okadaic acid (OKA) or with 1 mM sodium orthovanadate (NaORV) for 30 min at 37 °C. After incubation the cells were lysed and the proteins from 1.5×10^6 cells were separated by SDS/PAGE, blotted on Immobilon-P and assayed for kinase activity [16]. The arrowhead indicates the 60 kDa kinase.

Figure 3 Time-dependent inhibition of YT-INDY-CMC and kinetics of the appearance of cellular kinases

YT-INDY cells were incubated either alone or with 20 nM calyculin A or 1 μ M okadaic acid for the times indicated. Cells were then washed and assayed for cytotoxicity (A) or for kinase activity (B). Abbreviations used: CON, untreated controls; CAL, calyculin A-treated cells; OKA, okadaic acid-treated cells. The arrowhead indicates the 60 kDa protein kinase.

Figure 4 Stability of the 60 and 78 kDa kinases

YT-INDY cells were incubated with 20 nM calyculin A for 30 min at 37 °C. After incubation the cells were washed and reincubated in fresh medium, without calyculin A, for indicated times. Samples were collected and assayed for kinase activity. Abbreviations: C, untreated control cells; CAL, calyculin A-treated cells. The arrowhead identifies the 60 kDa kinase.

in calyculin A-treated cells. The 60 kDa band, however, showed intense kinase activity only in calyculin A-treated cells (Figure 2) but not in untreated or in cells treated with okadaic acid or sodium orthovanadate. In contrast, the 78 kDa protein band was present with basal activity in untreated as well as in okadaic acid-treated or sodium orthovanadate-treated cells (Figure 2). The inhibition of CTL-CMC by okadaic acid was fully active [11].

Kinetics of lysis inhibition and appearance of cellular kinase activity

To establish a correlation between the hyperphosphorylation of the 60 kDa protein and the loss of YT-INDY lytic activity, we examined the time course of appearance of this kinase after calyculin A treatment. We demonstrated that incubation of YT-INDY cells with 20 nM calyculin A for 5, 10, 15 and 30 minutes at 37 °C led to 14%, 26%, 77% and more than 98% inhibition of CMC respectively (Figure 3A). We also observed a parallel and gradual increase in the kinase activity associated with the 60 kDa protein (Figure 3B). The 60 kDa kinase appeared as early as 5 min and peaked at 30 min after calyculin A treatment. Thus our results indicate a possible association between the appearance of the 60 kDa kinase and the loss of YT-INDY-CMC.

Stability of 60 and 78 kDa kinases

To study the stability of the kinase activity associated with the 60 and 78 kDa proteins, we incubated YT-INDY cells with or without 20 nM calyculin A for 30 min at 37 °C, washed them and reincubated them in fresh medium without calyculin A. Samples were collected at different time points. Cells collected immediately after washing constituted the zero time point. We observed a gradual decline in the kinase activity. After 90 min the 60 kDa kinase had lost about 50% of its activity and after 2 h its activity was barely detectable. In contrast, the 78 kDa protein associated kinase activity remained stable and had lost only about 50% of its activity after 4 h (Figure 4). These results indicate that the 60 kDa protein has either a faster turnover/inactivation rate or is down-regulated faster than the 78 kDa protein.

To study the turnover of the kinase activity associated with the

Figure 5 Effect of cycloheximide treatment on calyculin A-sensitive kinases

YT-INDY cells were incubated without (CON) or with 30 μ g/ml cycloheximide (CHX) for 60 min at 37 °C followed by the addition of 20 nM calyculin A (CAL) as indicated. The cells were incubated for an additional 30 min, washed and assayed for kinase activity. The arrowhead indicates the 60 kDa kinase.

60 and 78 kDa proteins, we treated YT-INDY cells with 30 μ g/ml cycloheximide for 60 min at 37 °C and then added 20 nM calyculin A. The cells were then incubated for an additional 30 min at 37 °C and assayed for kinase activity. Cycloheximide treatment inhibited more than 98 % of protein synthesis (results not shown) but did not block the calyculin A-mediated activation of these kinases, indicating that they were expressed constitutively (Figure 5). The kinase activity of the 60 and 78 kDa proteins in cycloheximide-treated cells was approx. 50 % of the activity observed in untreated cells. This might be due either to a lack of new protein synthesis or to a fast turnover rate of these kinases.

Subcellular distribution and phosphoamino acid analysis of the 60 and 78 kDa kinase activity

To establish the cellular localization of these two kinases, we incubated YT-INDY cells with or without 20 nM calyculin A at 37 °C and performed subcellular fractionation as described previously [11]. The results showed a time-dependent increase in the kinase activity after calyculin A treatment (Figure 6). The results also revealed that the 78 kDa band was predominantly localized in the cytosolic fraction whereas the 60 kDa band was equally distributed between the membrane and the cytosolic compartments.

The phosphoamino acid analysis of the 78 and 60 kDa kinases (Figure 7) showed that the phosphorylation occurred at serine and threonine residues but not at tyrosine residues. These results further explain why incubation with up to 1 mM sodium orthovanadate did not activate the kinase activity associated with the 60 or 78 kDa protein (Figure 2). Moreover adding an anti-phosphotyrosine antibody to the cellular proteins extracted from the calyculin A-treated and okadaic acid-treated cells did not immunoprecipitate the 78 or 60 kDa proteins (results not shown). Taken together, these results suggest that the regulation of 60 and 78 kDa kinases is independent of tyrosine phosphorylation.

Effect of phorbol ester, ionophore and other pharmacological modulators on 60 and 78 kDa kinases

The Ser/Thr phosphorylation of the 60 and 78 kDa proteins after calyculin A treatment suggests that the inhibition of

Figure 6 Subcellular distribution of the 60 and 78 kDa kinases

YT-INDY cells were treated with 20 nM calyculin A for 30 min at 37 °C, washed and fractionated into cytosolic (CYTO) and membrane (MEMB) fractions as described [11]. The arrowhead indicates the 60 kDa kinase.

cytotoxicity might be linked to PKC, a diacylglycerol- and Ca^{2+} -dependent Ser/Thr kinase [21] implicated in signal transduction and in the regulation of the lytic activity of NK cells [2]. To test this possibility we first incubated YT-INDY cells with 10 or 100 ng/ml phorbol 12-myristate 13-acetate (PMA) for 2 h, added 20 nM calyculin A and reincubated the cells for an additional 30 min. The results showed that PMA did not activate these kinases or circumvent their activation after treatment with calyculin A (Figure 8A). PMA alone did not inhibit NK-CMC, whereas PMA and calyculin A added together inhibited YT-INDY lytic activity (results not shown).

To further confirm these observations and study the effect of Ca^{2+} on the appearance of the two kinases, we treated YT-INDY cells with 100 ng/ml PMA and 1 μ g/ml ionophore for 1 h at 37 °C and then added 20 nM calyculin A. The samples were reincubated for an additional 30 min at 37 °C. Incubation with PMA and ionophore did not affect the calyculin A-mediated activation of the 60 and 78 kDa kinases (Figure 8B). In addition, the presence or absence of extracellular Ca^{2+} did not significantly affect these kinases.

Treatment of YT-INDY cells with other inhibitors of secondary signals such as microcystin LR (a Ser/Thr phosphatase inhibitor), calphostin C and bisindolylmaleimide I, hydrochloride (BIS) (selective PKC inhibitors), wortmannin (an irreversible inhibitor of phosphatidylinositol 3-kinase), or genistein (a tyrosine kinase inhibitor) did not lead to the activation of either the 60 or 78 kDa kinase (Figure 8C). Treatment with sodium orthovanadate did not activate these kinases either (Figure 2). All these inhibitors, except microcystin LR and PMA, inhibited YT-INDY-CMC by more than 98 %. Calyculin A and okadaic acid were used as positive and negative controls respectively. Furthermore an activator (dibutyl-cAMP) and an inhibitor (HA-1004) of protein kinase A (PKA) had no effect on these kinases (results not shown). These results strongly suggest that the activation of the 60 and 78 kDa kinases is independent of PKC, PKA, tyrosine kinases or PTPases and that it is specific for calyculin A.

Characterization of the 60 and 78 kDa kinases by two-dimensional electrophoresis

Next, YT-INDY cells were treated with calyculin A for 30 min at 37 °C, washed and prepared for isoelectric focusing (IEF) as described [22]. The lysate proteins were separated, first by IEF

Figure 7 Phosphoamino acid analysis of the 78 and 60 kDa protein kinases

The 78 and 60 kDa kinase bands were excised, then digested with 6 M HCl for 60 min at 110 °C, and the phosphoamino acids were separated on cellulose plates by electrophoresis at 500 V for 70 min. The labels Ser, Thr and Tyr represent phosphoserine, phosphothreonine and phosphotyrosine; 1 and 2 indicate the 78 and the 60 kDa protein kinases respectively.

(first dimension) and then were further resolved (second dimension) by SDS/PAGE [15]. We observed only one band at 78 kDa whereas four bands were present at 60 kDa. These results suggest that the 78 kDa kinase has only one isoform whereas the 60 kDa kinase has four isoforms. The four isomeric forms of the 60 kDa kinase may represent different proteins or may be the same protein phosphorylated at different sites (Figure 9).

Effect of calyculin A on CD28-induced tyrosine phosphorylation

The role of tyrosine phosphorylation in the regulation of NK-CMC is well established [1–3]. In this study we show that calyculin A phosphorylates two proteins at Ser/Thr residues but not at tyrosine residues and that the 60 and 78 kDa kinases do not have any detectable phosphotyrosine. Further, to ascertain whether calyculin A mediates the inhibition of cytotoxicity by modulating the tyrosine kinase/phosphatase pathway, we studied the tyrosine phosphorylation in YT-INDY cells treated with anti-CD28. Because YT-INDY cells require CD28 for the recognition and killing of tumour target cells [15] we first treated the cells with calyculin A (20 nM) for 30 min at 37 °C, washed them twice with PBS at 4 °C then reincubated them with 5 µg/ml anti-CD28 antibodies on ice for 10 min. After incubation the cells were transferred to a 37 °C water bath and samples were collected at different time intervals. The samples, when analysed by Western blotting with a monoclonal antiphosphotyrosine antibody (4G10), revealed several minor and four major bands with altered profiles of phosphorylation (Figure 10). The induction of tyrosine phosphorylation in the 205 kDa protein peaked between 2 and 10 min and returned to control levels by 20 min. The 140 kDa protein showed a rapid (less than 2 min) induction in tyrosine phosphorylation that dephosphorylated at a slower rate. We also observed that anti-CD28 induced the tyrosine phosphorylation of a 120 kDa protein within 2 min of incubation. In contrast, calyculin A treatment completely dephosphorylated and blocked the anti-CD28-induced tyrosine phosphorylation of the 120 kDa protein. However, the anti-CD28-induced tyrosine phosphorylation of the 105 kDa protein was not affected by the calyculin A treatment (Figure 10). These

Figure 8 Effect of PMA, ionophore and other mediators on calyculin A-sensitive kinases

(A) YT-INDY cells were incubated without (CON) or with 10 and 100 ng/ml PMA as indicated for 2 h at 37 °C. After this first incubation, 20 nM calyculin A (CAL) was added as indicated and the cells were incubated for an additional 30 min at 37 °C, washed and assayed for kinase activity. The arrowhead indicates the 60 kDa kinase. (B) YT-INDY cells were first incubated with 100 ng/ml PMA and 1 µg/ml ionophore (IONO) for 2 h at 37 °C; then 20 nM calyculin A was added as required. The cells were reincubated for an additional 30 min. After incubation, cells were washed and assayed for kinase activity. (C) The YT-INDY cells were incubated with calyculin A (CAL; 20 nM), okadaic acid (OKA; 1 µM), PMA (100 ng/ml), BIS (5 µM), wortmannin (WORT; 5 µM) or genistein (GEN; 100 µg/ml) for 30 min, or with microcystin (MICR; 1 µM) and calphostin (CALPH; 200 nM) for 1 h at 37 °C. CON, control (no addition). The arrowhead indicates the 60 kDa kinase.

results suggest that calyculin A selectively inhibits the antigen-induced tyrosine phosphorylation of cellular proteins.

DISCUSSION

The regulation of phosphotyrosine kinases and phosphatases in the activation of CTL and NK cells is well documented [2–6] although the role of Ser/Thr kinases and related phosphatases is

Figure 9 Two-dimensional analysis of calyculin A-sensitive kinases

YT-INDY cells were treated with 20 nM calyculin A for 30 min at 37 °C, washed and lysed, and the proteins were separated by IEF in the first dimension and by SDS/PAGE in the second dimension as described by O'Farrell [22]. A, 78 kDa kinase; B, 60 kDa kinase. The arrowheads indicate the 60 kDa kinase isoforms.

Figure 10 Regulation of antigen-induced tyrosine phosphorylation in YT-INDY cells by calyculin A

YT-INDY cells were incubated either alone (—) or with (+) 20 nM calyculin A (CAL) for 30 min at 37 °C. Cells were then washed and incubated without (—) or with (+) 5 µg/ml anti-CD28 antibodies (x-CD28) on ice for 10 min. After incubation, cells were transferred to 37 °C and samples were collected at the indicated times. Arrows indicate 105, 120 and 140 kDa protein bands.

still poorly understood. Recently we have established that the inhibition of Ser/Thr phosphatases leads to the functional inactivation of CTL and NK cells [11]. The different effects of calyculin A and okadaic acid on the lytic activity of CTL and NK cells [11] provided a new approach to define the role of Ser/Thr phosphatases further in tumour cell destruction. In this study we have investigated the effect of calyculin A and okadaic acid, two Ser/Thr phosphatase inhibitors, on the lytic activity of an NK-like cell line YT-INDY.

Okadaic acid and calyculin A are selective and potent inhibitors of PP1 and PP2A protein phosphatases and their role in regulating NK-CMC [11] might depend on their relative strengths in inhibiting different pools of cellular phosphatases [23]. Okadaic acid inhibits the purified catalytic subunit of PP2A 50–100 times more effectively than PP1, whereas calyculin A inhibits PP1 at least 10-fold more effectively than okadaic acid [24]. Despite their unrelated structures, calyculin A binds to the okadaic acid receptors [25]. Calyculin A binds to the particulate fraction with

a 10-fold stronger affinity than okadaic acid, whereas both compounds show the same affinity for the cytosolic fractions of purified phosphatases [25,26]. Okadaic acid acts as a non-competitive or mixed inhibitor for restricted types of sensitive phosphatases and inhibits by binding to a site different from the substrate-binding site [27]. Thus the relative effectiveness of okadaic acid and calyculin A in regulating a Ser/Thr phosphatase-sensitive process *in vivo* depends on the type and the subcellular distribution of the phosphatases [26,28]. The nature and the distribution of Ser/Thr phosphatases in NK cells is not well characterized. Their differential localization might explain the relative effectiveness of okadaic acid and calyculin A in regulating NK-CMC. Further work to characterize these calyculin A-sensitive and okadaic acid-sensitive phosphatases is in progress.

To study the effect of Ser/Thr phosphatase inhibition on cellular kinases we used an in-gel kinase assay, a well-established assay for MAP and other kinases [16–20]. Treatment of YT-INDY cells with calyculin A led to the rapid hyperphosphorylation of a 60 kDa and a 78 kDa protein (Figure 2). The appearance of these bands is specific for calyculin A treatment, as okadaic acid (1 µM) and sodium orthovanadate (1 mM) had no effect (Figure 2). In addition, the PKC activator PMA, the PKC inhibitors calphostin C and BIS, the tyrosine kinase inhibitor genistein and the Ser/Thr phosphatase inhibitor microcystin LR all failed to phosphorylate the 60 and 78 kDa proteins (Figure 8). The PKA activator dibutyryl-cAMP and the PKA inhibitor HA-1004 also had no effect on the phosphorylation of these proteins (results not shown). Microcystin LR is a potent inhibitor of PP1 and PP2A phosphatases *in vitro*; however, in cells it has biochemical characteristics and specific activities similar to those of okadaic acid [29,30]. We confirmed these findings and observed that microcystin LR affected NK cell-CMC and the activation of kinases in a manner analogous to okadaic acid (Figure 8C). Our results suggest that the inhibition of YT-CMC by calyculin A is an early event, upstream of the secondary signal transduction by PKC, PKA and tyrosine kinases and specific for calyculin A-sensitive phosphatases.

The kinetics of calyculin A-mediated inhibition of YT-INDY and the phosphorylation of the 60 and 78 kDa proteins showed a correlation between the two phenomena (Figure 3). We did not observe any precursor product relationship between the 78 and 60 kDa proteins, suggesting that they are different and that they might have different effects on the lytic process. The specific time-dependent phosphorylation of the 60 kDa protein with the parallel loss of lytic activity suggests that the two events might be related (Figure 3). Studies on the stability of these two proteins further revealed that the 78 kDa protein has a longer half-life than the 60 kDa protein (Figure 4). Cycloheximide treatment showed that the activated 60 kDa protein had a faster down-regulation than the 78 kDa protein, further indicating that these proteins were constitutively present in an inactive form in YT-INDY (Figure 5). The subcellular fractionation study suggests that the 78 kDa protein is localized in the cytosolic compartment whereas the 60 kDa protein seems to be equally distributed between the membrane and cytosolic compartments (Figure 6). Two-dimensional analysis of cellular lysates revealed that the 78 kDa protein existed as a single band whereas the 60 kDa band showed four isomeric forms. This might be a consequence of phosphorylation at four different sites because this procedure can separate proteins that differ even at one phosphorylation site [22].

Our results also indicate that calyculin A and okadaic acid activate separate biochemical pathways leading to different outcomes in YT-INDY cell function. Several lines of evidence

suggest that the 60 and 78 kDa proteins can be considered to be autophosphorylated kinases (phosphotransferases). First, the cell lysates were initially resolved by reducing SDS/PAGE, transferred to a poly(vinylidene difluoride) membrane and only then was the assay performed. This rules out the possibility that the two proteins are phosphorylated by another kinase. Secondly, these two proteins have the capability to transfer γ -phosphate from ATP to the Ser/Thr residues of the substrates (proteins). On the basis of the nature of the experimental protocol the protein bands observed at 60 and 78 kDa might represent monomeric kinases either autophosphorylating themselves or phosphorylating neighbouring substrates. To rule out the possibility that these proteins are phosphorylated by neighbouring kinases, cellular proteins were first separated by IEF and then by reducing SDS/PAGE in a second dimension: the same 60 and 78 kDa phosphorylated bands were still observed (Figure 9). These results do not support the notion of the phosphorylation of neighbouring proteins because it seems highly unlikely that two neighbouring proteins would have identical isoelectric points and identical molecular masses. These results strongly suggest that these two proteins have an intrinsic kinase activity and that they autophosphorylate in response to calyculin A treatment. However, we cannot rule out the involvement of other kinases in YT-CMC that are either hard to renature or require oligomeric structure to be active. The 60 and 78 kDa proteins might also represent autophosphorylated catalytic subunits of oligomeric kinases.

Several Src-family tyrosine kinases including p56^{lck}, p59^{lyn} and p62^{c-yes} are involved in NK-cell activation [31]. Watts et al. [32] have shown that stimulation of T lymphocytes by IL-2 phosphorylates p56^{lck} at a serine residue and this changes its electrophoretic mobility to 60 kDa (p60^{lck}). It is unlikely that the 60 kDa kinase reported in this study belongs to this Src family because Src kinases show strong phosphorylation at tyrosine residues and we did not detect any phosphorylated tyrosine on the two kinases described here (Figure 7). Moreover, sodium orthovanadate or genistein did not affect the expression of these kinases (Figures 2 and 8) and also they could not be detected either by immunoprecipitation or by Western blot analysis with antiphosphotyrosine antibodies (results not shown). In addition, the lack of any detectable phosphotyrosine on the 60 kDa kinase also excludes this protein from being a member of the MAP kinase family because MAP kinases require phosphorylation at both the tyrosine and the Ser/Thr residues for their activity [33,34].

Treatment of YT-INDY cells with calyculin A changes the level of tyrosine phosphorylation in cellular proteins (Figure 10). We have also observed decreased tyrosine kinase activity in response to calyculin A treatment (results not shown). These observations further strengthen our contention that Ser/Thr and tyrosine kinase pathways are equally important in NK-cell lytic activity and that these kinases 'cross-talk' during the regulation of CMC [11]. We did not observe any significant effect of okadaic acid on any of these processes, substantiating our earlier observation that calyculin A and okadaic acid have differential effects on the regulation of cytotoxicity mediated by CTL and NK cells [11].

In conclusion, the present study provides new insight into the differential modes of action of calyculin A and okadaic acid, and establishes an important role for Ser/Thr phosphatases in NK-cell-mediated tumour cell lysis. In addition we have identified two Ser/Thr kinases that seem to be regulated by calyculin A-

sensitive phosphatases that ultimately control the lytic function of an NK-like cell line, YT-INDY. Finally, the appearance of the 60 and 78 kDa Ser/Thr kinases is independent of PKC activation and the calyculin A-sensitive Ser/Thr phosphatases seem also to regulate the selective tyrosine kinase/phosphatase pathways.

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REFERENCES

- Trinchieri, G. (1989) *Adv. Immunol.* **47**, 187–375
- Brahmi, Z. (1992) in *NK Cell-mediated Cytotoxicity: Receptors, Signaling and Mechanisms* (Lotzova, E. and Herberman, R., eds.), pp. 117–128, CRC Press, Boca Raton, FL
- Leibson, P. J. (1992) in *NK Cell-mediated Cytotoxicity: Receptors, Signaling and Mechanisms* (Lotzova, E. and Herberman, R., eds.), pp. 129–146, CRC Press, Boca Raton, FL
- Podack, E. R. and Kupfer, A. (1991) *Annu. Rev. Cell Biol.* **7**, 479–504
- Purimutter, R. M., Levin, S. D., Appelby, M. W., Anderson, S. J. and Alberola-Ila, J. (1993) *Annu. Rev. Immunol.* **11**, 451–499
- Alexander, D., Shiroo, M., Robinson, A., Bitten, M. and Shivan, E. (1992) *Immunol. Today* **13**, 477–481
- Yamada, A., Streuli, M., Rothstein, D. M., Schlossman, S. F. and Morimoto, C. (1990) *Eur. J. Immunol.* **20**, 1655–1660
- Tonks, N. K., Diltz, E. H. and Fischer, E. H. (1990) *J. Biol. Chem.* **265**, 10674–10680
- Valentine, M. A., Widmer, M. B., Ledbetter, J. A., Pinault, F., Voice, R., Clark, E. A., Gallis, B. and Brautigan, D. L. (1991) *Eur. J. Immunol.* **21**, 913–919
- Ostergaard, H. and Trowbridge, I. S. (1991) *Science* **253**, 1423–1425
- Bajpai, A. and Brahmi, Z. (1994) *J. Biol. Chem.* **269**, 18864–18869
- Yodoi, J., Teshigawara, K., Nikaide, T., Fukui, K., Noma, T., Honjo, T., Takigawa, M., Sasaki, M., Minato, N., Tsudo, M., Uchiyama, T. and Maeda, M. (1985) *J. Immunol.* **134**, 1623–1630
- Montel, A. H., Morse, P. A. and Brahmi, Z. (1995) *J. Cell. Immunol.* **160**, 104–114
- Bajpai, A. and Brahmi, Z. (1993) *Biochim. Biophys. Acta* **1177**, 291–298
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Ferrell, Jr., J. E. and Martin, G. S. (1991) *Methods Enzymol.* **200**, 430–435
- Ferrell, Jr., J. E. and Martin, G. S. (1990) *Mol. Cell. Biol.* **10**, 3020–3026
- Lee, H., Ghose-Dastidar, J., Winawer, S. and Friedman, E. (1993) *J. Biol. Chem.* **268**, 5255–5263
- Tobe, K., Kadowaki, T., Hara, K., Gotoh, Y., Kosako, H., Matsuda, S., Tamemoto, H., Ueki, K., Akanuma, Y., Nishida, E. and Yazaki, Y. (1992) *J. Biol. Chem.* **267**, 21089–21097
- Kameshita, I. and Fujisawa, H. (1989) *Anal. Biochem.* **183**, 139–143
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
- Fujiki, H. and Suganuma, M. (1993) *Adv. Cancer Res.* **61**, 143–194
- Suganuma, M., Fujiki, H., Okabe, S., Nishiwaki, S., Brautigan, D., Ingebritsen, T. S. and Rosner, M. R. (1992) *Toxicol.* **30**, 873–878
- Suganuma, M., Suttajit, M., Suguri, H., Ojika, M., Yamada, K. and Fujiwaki, H. (1989) *FEBS Lett.* **250**, 615–618
- Suganuma, M., Fujiki, H., Furuya-Suguri, H., Yoshizawa, S., Kato, Y., Fusetani, N. and Sugimura, T. (1990) *Cancer Res.* **50**, 3521–3525
- Bialojan, C. and Takai, A. (1988) *Biochem. J.* **256**, 283–290
- Fujiki, H., Suganuma, M., Nishiwaki, S., Yoshizawa, S., Yatsunami, J., Matsushima, R., Furuya, H., Okabe, S., Matsunaga, S. and Sugimura, T. (1992) in *Relevance of Animal Studies to the Evaluation of Human Cancer Risk* (D'Amato, R., Salga, T. J., Farland, W. and Henry, C., eds.), pp. 337–350, Wiley-Liss, New York
- Matsushima, R., Yoshizawa, S., Watanabe, M. F., Harada, K.-I., Furusawa, M., Carmichael, W. W. and Fujiki, H. (1990) *Biochem. Biophys. Res. Commun.* **171**, 867–874
- Yoshizawa, S., Matsushima, R., Watanabe, M. F., Harada, K.-I., Ichihara, A., Carmichael, W. W. and Fujiki, H. (1990) *J. Cancer Res. Clin. Oncol.* **116**, 609–614
- Eiseman, E. and Bolen, J. B. (1990) *Cancer Cells* **2**, 303–309
- Watts, J. D., Welham, M. J., Kalt, L., Schrader, J. W. and Aebersold, R. (1993) *J. Immunol.* **151**, 6862–6871
- Anderson, N. G., Maller, J. L., Tonks, N. K. and Sturgill, T. W. (1990) *Nature (London)* **343**, 651–653
- Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J. and Sturgill, T. W. (1991) *EMBO J.* **10**, 885–892