Identification on melanoma cells of p39, a cysteine proteinase that cleaves C3, the third component of complement: amino-acid-sequence identities with procathepsin L

Didier JEAN, Jacques HERMANN, Fernando RODRIGUES-LIMA, Monique BAREL, Michelle BALBO and Raymond FRADE*

Immunochimie des Régulations Cellulaires et des Interactions Virales, INSERM U.354, Centre INSERM, Hôpital Saint-Antoine, 75012 Paris, France

We previously identified, on normal or tumour cells, two membrane proteinases, p57 and p65, that cleave human C3, the third component of complement, thus regulating C3's biological properties. Whereas p57 was purified from human erythrocytes, p65 was identified using polyclonal anti-p57 antibodies on a human melanoma cell line resistant to complement lysis. Analysis of cell distribution of C3-cleaving proteinases established that DSm, a murine melanoma cell line, expressed a C3-cleaving proteinase distinct from p57 and p65 proteinases. Thus we purified the C3-cleaving proteinase solubilized from membranes of DSm cells. The purified proteinase, termed 'p39' on the basis of its molecular mass of 39 kDa, was identified, using specific proteinase inhibitors, as a cysteine proteinase. Anti-p39 antibodies, prepared against highly purified p39, localized the

p39 C3-cleaving proteinase mainly at the cell surface and demonstrated that p39 is also secreted. Anti-p39 antibodies inhibited solubilized C3-cleaving activity. Preincubation of DSm cells with anti-p39 F(ab')₂ fragments increased up to 60 % complement cell susceptibility. Amino acid analysis of N-terminal and three other regions of p39 demonstrated that this C3-cleaving proteinase carries 100 % identity within four regions of procathepsin L. This is the first demonstration that a melanoma cell line expresses on its surface and secretes a p39 C3-cleaving cysteine proteinase that shares sequence identities with procathepsin L. Thus the p39 cysteine proteinase represents a new member of the C3-cleaving proteinase family associated with, and/or expressed on, the cell surface.

INTRODUCTION

Human C3 is the pivotal component of complement whose activation, through classical or alternative pathways, leads to cell lysis [1]. The first step of C3 activation in C3b allows covalent deposition of this fragment on the cell surface and thus formation of membrane-attack complex on the target cell. In addition, human C3 is involved in cell regulation. Indeed, during C3 activation, different fragments, namely C3a, C3b, C3bi, C3dg and C3d, are generated [2] that could interact with specific cell-surface C3 receptors, namely C3aR, CR1, CR3, CR4 and CR2 respectively [3], thus triggering cell regulation [4–6]. In addition to the C3 receptors, C3 fragments also interact with delay-accelerating factor ('DAF') [7], membrane cofactor protein ('MCP') [8], p57 [9,10] and p65 [11], only the last two membrane proteins carrying proteinase activity against C3.

Thus cell-surface proteinases that cleave C3 could on the one hand protect normal or tumour target cells against autologous complement lysis by preventing deposition of activated C3b on their surface and on the other hand generate fragments and peptides which exert regulatory functions on normal and tumour cells.

P57 is a membrane serine proteinase identified and purified from human erythrocyte membranes [9,10]. Recently we demonstrated that p57 is a fragment of ankyrin, a peripheral membrane protein involved in the spectrin-actin network and which mediates the attachment of the erythrocyte skeleton to the plasma membrane [12]. Ankyrin binds simultaneously to spectrin and to the cytoplasmic domain of the plasma-membrane anion transporter [13,14]. Determination of amino acid sequence of the

p57 proteinase and analysis of immunological properties and C3-cleaving activity of ankyrin fragments demonstrated that the p57 C3-cleaving proteinase is generated from the 62 kDa spectrin-binding domain of erythrocyte ankyrin.

In addition, using polyclonal anti-p57 antibodies (Ab), prepared against purified p57 proteinase, we also demonstrated the expression of p65, a membrane proteinase characterized by a molecular mass of 65 kDa, with C3-cleaving activity and antigenically related to p57, at the surface of a human melanoma cell line resistant to complement [11]. P65 C3-cleaving proteinase contributed at least 50 % to the resistance of these tumour cells to human complement lysis. No C3-cleaving proteinase was expressed at the surface of melanoma cell lines susceptible to complement lysis [11]. The role of the p65 C3-cleaving proteinase in melanoma-cell resistance to the humoral immune system led us to analyse the cell distribution of C3-cleaving proteinases in murine and human melanoma cells. Among the cell lines tested, some expressed the p65 proteinase and others expressed C3-cleaving activity neither related to p57 [10] nor to p65 [11] proteinases (D. Jean, J. Hermann, F. Rodrigues and R. Frade, unpublished work).

In the present report we purified from murine melanoma cells a new C3-cleaving proteinase ('p39') characterized by a molecular mass of 39 kDa. By using proteinase inhibitors, we demonstrated that p39 proteinase is a cysteine proteinase. Amino acid analysis of the N-terminus and three regions of the purified p39 demonstrated that this C3-cleaving proteinase carries 100 % amino-acid-sequence identity with four regions of procathepsin L. By using anti-p39 antibodies, prepared against highly purified p39, we demonstrated that C3-cleaving proteinase is expressed on the

Abbreviations used: E-64, *N*-(L-3-carboxy-2,3-*trans*-carboxyoxirane-2-carbonyl)-L-leucyl-(4-guanidino)butane; Ab, antibody; C3, third component of complement.

* To whom correspondence should be sent.

cell surface and is also secreted in the extracellular medium. Thus p39 is a cysteine proteinase that carries amino-acid-sequence identities with procathepsin L and represents another member of the C3-cleaving proteinase family expressed on surface of melanoma cells.

MATERIALS AND METHODS

Materials

C3 was purified from fresh plasma as described by Hammer et al. [15]. Protein A-Sepharose CL-4B, PD-10, DEAE-Sepharose CL-6B, Mono-Q HR 5/5, Mono-S HR 5/5, phenyl-Superose HR 5/5, Superose 12 columns were purchased from Pharmacia (Uppsala, Sweden). Electrophoresis reagents, molecular-mass marker proteins and silver-staining kit were from Bio-Rad (Hercules, CA, U.S.A.). Dimethyl pimelimidate, *N*-(L-3-carboxy-2,3-*trans*-carboxyoxirane-2-carbonyl-L-leucyl-(4-guanidino)butane (E-64; a specific cysteine-proteinase inhibitor), iodoacetic acid and leupeptin (two cysteine/serine proteinase inhibitors), 3,4-dichloroisocoumarin and PMSF (two serine-proteinase inhibitors), 1,10-phenanthroline and EDTA (two metalloproteinase inhibitors), pepstatin A (an aspartic-proteinase inhibitor) and CHAPS were from Sigma (St. Louis, MO, U.S.A.). Bicinchoninic acid ('BCA') protein assay kit was from Pierce (Rockford, IL, U.S.A.). Calibration proteins for gel filtration were from Boehringer (Mannheim, Germany). Kanamycin and fetal-calf serum were from Eurobio (Les Ulis, France). Other culture reagents were from Seromed (Berlin, Germany).

Cells

The cell lines used were the murine DSm melanoma cell line and the human melanoma cell lines 3115 and DX3, kindly provided by Dr. M. Bar-Eli (M. D. Anderson Cancer Center, Houston, TX, U.S.A.). Cells were grown as monolayers in RPMI 1640 medium containing 10% fetal-calf serum, 100 µg/ml streptomycin, 100 units/ml penicillin, 100 µg/ml kanamycin and 2 mM glutamine, at 37 °C in a 5%-CO₂ incubator.

Preparation of total membrane extracts

Confluent cells were washed with PBS, trypsin-treated, then washed three times by centrifugation at 500 *g* for 10 min. Harvested cells were stored at -80 °C. Cells were disrupted by thawing in PBS and then submitted to a 300 *g* centrifugation for 10 min to remove nuclei and undisrupted cells. The supernatant was submitted to a 100 000 *g* centrifugation for 30 min to collect total membranes. Membranes were washed in PBS, then solubilized in PBS containing 10 mM CHAPS for 2 h. Solubilized membrane components were collected by centrifugation at 100 000 *g* for 30 min and stored at -80 °C. The residual membrane pellet was extracted again in a similar manner. Protein concentration was determined by the method of Smith et al. [16], using the bicinchoninic acid reagent. Proteins contained in samples were analysed by SDS/PAGE [17]. Gels were stained with silver reagent.

Radiolabelling

A 100 µg portion of purified C3 or protein A were labelled with ¹²⁵I (648 mCi/mmol; carrier-free; Amersham, Les Ulis, France) using immobilized Iodogen (Pierce) [18]. Unbound ¹²⁵I was removed by filtration on a PD-10 column. The specific radioactivity was 10⁶ c.p.m./µg.

C3-cleaving activity in membrane extracts and on cells

A 70 µg portion of solubilized membrane components or samples from columns were incubated with 10 µl of ¹²⁵I-C3 (15000 c.p.m.) for 2 h at 37 °C. For inhibition assays, solubilized membrane components or 0.2 µg of purified 39 kDa protein were preincubated with 5 µl of proteinase inhibitors at the appropriate concentration or with purified IgG of anti-p39 Ab, for 1 h at 4 °C before incubation with ¹²⁵I-C3. Then samples were submitted to SDS/PAGE under reducing conditions. Gels were autoradiographed on Trimax XD films (3M, Paris, France) with intensifying screens. The C3 cleavage was characterized by generation of a 42 kDa fragment identified as C3dg by using a monoclonal anti-C3d Ab prepared in our laboratory (A. Fiandino, M. Barel and R. Frade, unpublished work). This fragment was quantified using a laser densitometer (2202 Ultrosan; LKB, Uppsala, Sweden), equipped with an integrator (D-2000; Merck, Darmstadt, Germany). The percentage of C3 cleavage was determined as the ratio of the amount of C3dg fragment generated to the total C3 radioactivity. One unit of activity was defined as 1% of C3dg fragment generated by 1 ml of sample in 1 h at 37 °C. The C3-cleaving activity of the purified proteinase was also measured with unlabelled C3 by detecting C3 fragments with polyclonal anti-C3 antibodies in an immunoblotting assay, as previously described [19]. Furthermore, C3-cleaving activity was also measured on DSm cells. Cells (10⁶) were incubated, in a total volume of 50 µl, with 10 µl of ¹²⁵I-C3 for 60 min at 37 °C. After centrifugation at 300 *g* for 5 min, the labelled components contained in supernatant were analysed by SDS/PAGE under reducing conditions, as described above.

Purification of C3-cleaving activity

Purification of p39 C3-cleaving proteinase was carried out at 4 °C, using fast-protein-liquid-chromatographic columns monitored by an LKB HPLC controller system.

DEAE-Sepharose CL-6B chromatography

Solubilized membrane components were dialysed against 20 mM Tris/HCl, pH 7.5, containing 1 mM CHAPS (buffer A) and applied to a DEAE-Sepharose CL-6B column (1.5 cm × 12 cm), equilibrated with this buffer, at a flow rate of 0.6 ml/min. The column was washed with 30 ml of buffer A and eluted with a linear gradient of 0–1 M NaCl in 150 ml of buffer A. Fractions (1.2 ml each) were collected. Fractions containing C3-cleaving activity were pooled and dialysed against 20 mM Tris/HCl buffer, pH 7, containing 1 mM CHAPS (buffer B).

Mono-Q

Dialysed samples from the previous step were loaded, at a flow rate of 1 ml/min, on to a Mono-Q HR 5/5 column equilibrated with buffer B. Proteins were eluted using a linear gradient of 0–0.5 M NaCl in buffer B. Fractions (500 µl each) were collected. Samples containing C3-cleaving activity were pooled and dialysed against 50 mM Na₂HPO₄ buffer, pH 6, containing 1 mM CHAPS (buffer C).

Mono-S

Active fractions isolated from the Mono-Q column were applied at a flow rate of 1 ml/min to a Mono-S HR 5/5 column equilibrated with buffer C. Proteins were eluted using a linear gradient of 0–0.5 M NaCl in buffer C. C3-cleaving activity was

recovered in a main peak which was dialysed against 50 mM Na_2HPO_4 buffer, pH 6.9, containing 1.3 M $(\text{NH}_4)_2\text{SO}_4$ and 1 mM CHAPS (buffer D).

Phenyl-Superose

The dialysed solution from the previous step was applied to a phenyl-Superose HR 5/5 column equilibrated with buffer D at a flow rate of 0.5 ml/min. After washing with the same buffer until the A_{280} reached the baseline, the column was eluted with a reverse linear gradient from 1.3 to 0 M $(\text{NH}_4)_2\text{SO}_4$. The C3-cleaving activity was eluted from this column in one peak. Fractions containing C3-cleaving activity were dialysed against 50 mM Na_2HPO_4 buffer, pH 7, with 1 mM CHAPS and then stored at -80°C for further studies.

Anti-p39 Ab preparation

A 1 μg portion of p39 C3-cleaving proteinase purified on Superose 12 was mixed with an equal volume of complete Freund's adjuvant and injected into rabbits. Three additional injections of 1 μg of purified p39 were processed at 2-week intervals: the first was mixed with complete Freund's adjuvant and the two others mixed with incomplete Freund's adjuvant. IgG fractions were isolated by 45 %-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitation, purified on Protein A-Sepharose and analysed on purified p39 by immunoblotting techniques, using ^{125}I -Protein A as previously described [19]. F(ab')_2 fragments were prepared from anti-p39 Ab or preimmune serum immunoglobulins of the same rabbit, as previously described [11].

Immunoabsorption of C3-cleaving activity

IgG fractions of sera (0.3 mg) were immobilized on 10 mg of Protein A-Sepharose CL-4B. Immunoglobulins bound on Protein A were cross-linked using 50 mM dimethyl pimelimidate, as described by Schneider et al. [20]. Immunobeads obtained were washed with PBS containing 10 mM CHAPS, then incubated with solubilized membrane components for 90 min at 4°C . After centrifugation at 1000 g for 3 min at 4°C , effluents were tested for C3-cleaving activity.

Immunofluorescence

DSm cells (2×10^5) were resuspended in 100 μl of PBS, smeared on a slide and air-dried. The cells were then fixed on the slides with 4% paraformaldehyde in PBS for 10 min at room temperature. The slides were washed three times in PBS, followed by a 10 min incubation in PBS containing 50 mM NH_4Cl . Subsequent washes and antibody dilutions were performed in PBS containing 0.2% BSA. Slides were incubated for 30 min with 0.1 mg of the IgG fraction of anti-p39 Ab or preimmune serum, washed twice and then incubated for 30 min with 100 μl of rhodamine-conjugated pig anti-rabbit IgG (Dako, Glostrup, Denmark) diluted 1:50. After three washings, cells were analysed using a fluorescence microscope (model BH-2; RFCA Olympus, Rungis, France). Photographs were taken with a 35 mm Olympus camera.

Preparation of DSm-cell-conditioned medium

Cells were grown as monolayer in culture flasks. At 70–80% confluence, cells were washed four times in RPMI containing 100 mg/ml streptomycin, 100 units/ml penicillin, 100 mg/ml kanamycin and 2 mM glutamine to remove residual fetal-calf serum. Then cells were grown for the indicated times in 3 ml of this

serum-free medium. Conditioned media were centrifuged at 400 g for 10 min and filtered on a 0.2 mm-pore-size filter (Sartorius, Göttingen, Germany), then concentrated 12-fold on Ultrafree-MC 10,000 NMWL Millipore filter and dialysed against PBS. Samples were stored at -80°C .

Cytotoxicity assay

Mouse DSm cells were harvested mechanically and washed with complete medium. Cells were labelled with ^{51}Cr for 3 h at 37°C . After four washes in RPMI, 5×10^4 DSm cells per well were preincubated with 10 μg of F(ab')_2 fragments for 45 min at 4°C . Then, 20 μl of monoclonal anti-H2 Ab (clone 34-1-2S; kindly provided by Doctor Marika PLA, INSERM U.93, Paris, France), were added for 30 min at 4°C , followed by the addition of 50 μl of rabbit complement. After 1 h at 37°C , plates were centrifuged at 300 g for 5 min and radioactivity released in supernatant was counted in a γ -radiation counter (Beckman).

RESULTS

C3-cleaving activity in melanoma cells

C3-cleaving activity was tested by incubating human ^{125}I -C3 with a constant amount of solubilized membrane components prepared from different murine and human melanoma cell lines. C3-cleaving activity was measured by monitoring the generation of C3dg fragment. Results showed that the different cell lines could be divided in three groups characterized by very high, medium/low and very low C3-cleaving activity. One murine DSm and two human 3115 and DX3 cell lines were representative of these three groups. C3-cleaving activities in the DSm cell line was 3- and 10-fold higher than in 3115 and DX3 cell lines respectively. Preliminary analysis suggested that the C3-cleaving activity carried by DSm cell line was neither related to the p57 [10] nor to the p65 [11] proteinases. Thus DSm cells were chosen to purify this C3-cleaving activity.

Purification of the C3-cleaving activity from DSm melanoma cells

Membrane components were solubilized in 10 mM CHAPS using total membranes prepared from 4×10^9 DSm murine cells. Solubilized membrane components were submitted to steps of purification, in buffers always containing 1 mM CHAPS. C3-cleaving activity of each sample was measured as described above. Representative profiles of the columns are shown in Figures 1(a)–1(d).

In the first step, solubilized membrane components were applied to a DEAE-Sepharose CL-6B column, equilibrated with buffer A, on which C3-cleaving activity was retained. This proteinase activity was eluted from the column between 0.26 and 0.39 M NaCl using a linear gradient (Figure 1a). Fractions containing C3-cleaving activity isolated from DEAE-Sepharose CL-6B were pooled, dialysed against buffer B and applied on to a Mono-Q column. The C3-cleaving activity was eluted between 0.15 and 0.32 M NaCl using a linear gradient (Figure 1b). Active fractions isolated from Mono-Q were pooled, dialysed against buffer C and applied on to a Mono-S column. The C3-cleaving activity was eluted between 0.16 and 0.29 M NaCl using linear gradient (Figure 1c). The samples containing C3-cleaving activity were pooled, dialysed against buffer D, then submitted to phenyl-Superose column chromatography. C3-cleaving activity was eluted between 0.78 and 0.65 M $(\text{NH}_4)_2\text{SO}_4$ using a reverse linear gradient (Figure 1d). The different steps of purification are summarized in Table 1. Determination of protein concentration and specific activity allowed one to calculate a purification factor

Figure 1 Purification of C3 cleaving-proteinase

(a) Chromatography on a DEAE-Sepharose column. Total membrane extracts were prepared from 4×10^9 DSm cells. Solubilized membrane components were then chromatographed on DEAE-Sepharose CL-6B. Proteins were eluted with a linear gradient of 0–1 M NaCl in buffer A. (b) Chromatography on a Mono-Q HR 5/5 column. The peak of C3-cleaving activity isolated from the DEAE-Sepharose column was applied to a Mono-Q column and proteins were eluted with a linear gradient of 0–0.5 M NaCl in buffer B. (c) Chromatography on a Mono-S HR 5/5 column. The peak of C3-cleaving activity isolated from the Mono-Q column was applied to a Mono-S column and proteins were eluted by a linear gradient of 0–0.5 M NaCl in buffer C. (d) Chromatography on a phenyl-Superose HR 5/5 column. The peak of C3-cleaving activity isolated from Mono-S column was applied to a phenyl-Superose column, and proteins were eluted by a reverse linear gradient of 1.3–0 M $(\text{NH}_4)_2\text{SO}_4$ in buffer D. Each column profile is representative of one of four separate experiments. In each chromatography, samples were tested in C3-cleaving activity following ^{125}I -C3 cleavage, analysed by SDS/10%-PAGE under reducing conditions and by autoradiography. Relative activity was calculated by considering the most active fraction as 100%.

Table 1 Purification of C3-cleaving proteinase from membranes of DSm melanoma cells

Step	Volume (ml)	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Membrane extract	169.00	327.00	4872	15	100	1
DEAE-Sepharose	19.00	36.00	3947	110	81	7
Mono-Q	6.40	6.70	3508	524	72	35
Mono-S	2.50	0.43	2101	4886	43	328
Phenyl-Sepharose	1.77	0.04	976	24400	20	1638

Figure 2 Purified C3-cleaving proteinase

(a) SDS/PAGE. Samples having C3-cleaving activity and purified from the phenyl-Superose column were analysed by SDS/12%-PAGE under reducing (lane 1) or non-reducing (lane 2) conditions. Proteins were detected by silver staining. Molecular-mass markers (kDa) are indicated. (b) Cleavage of unlabelled C3. Unlabelled C3 was incubated with buffer (lane 1) or purified p39 proteinase (lane 2), for 30 min at 37 °C. Then samples were submitted, under reducing conditions, to SDS/PAGE analysis. Proteins were electrotransferred, then immunoblotted using polyclonal anti-C3 Ab. Bound Ab were detected with 125 I-Protein A.

of 1638 and a final yield of 40 μ g from 327 mg of total solubilized proteins (Table 1). Analysis by SDS/PAGE and silver staining of samples containing the purified enzyme demonstrated (Figure 2a) the presence of a single band characterized, under reducing (lane 1) and non-reducing (lane 2) conditions, by a molecular mass of 39 kDa. This purified proteinase cleaved 125 I-labelled C3 (Figure 1), as well as unlabelled C3 (Figure 2b). Thus this proteinase was called 'p39 C3-cleaving proteinase'.

Identification of C3-cleaving proteinase

The class of the p39 C3-cleaving proteinase was determined by preincubating the purified enzyme, as well as the total membrane extracts, with proteinase inhibitors (Table 2). The C3-cleaving activity of purified p39 was strongly inhibited by 0.01 mM E-64, a specific cysteine-proteinase inhibitor. In addition, 1 mM iodoacetic acid and 0.01 mM leupeptin, which are also cysteine-proteinase inhibitors, inhibited p39 C3-cleaving activity totally. However, 0.1 mM 3,4-dichloroisocoumarin, 1 mM PMSF, 10 mM 1,10-phenanthroline, 5 mM EDTA and 0.01 mM pepstatin A did not inhibit the p39 C3-cleaving activity. The identical effect of these proteinase inhibitors on the purified p39 activity as well as on C3-cleaving activity present in solubilized membrane components (Table 2) supported the notion that this C3-cleaving

Figure 3 Immunoblotting analysis of p39 C3-cleaving proteinase

A 1.3 μ g portion of purified p39 proteinase (lanes 1 and 3) or 10 μ g of human C3 (lane 2) were submitted to SDS/10%-PAGE under reducing conditions, then electrotransferred on to cellulose nitrate sheets. Membranes were incubated with IgG fractions of rabbit anti-p39 Ab (lanes 2 and 3) or preimmune serum (lane 1), then incubated with 125 I-Protein A. Molecular-mass markers (kDa) are indicated.

activity is carried by only one proteinase, namely p39, which is a cysteine proteinase.

Immunological analysis of anti-p39

Polyclonal anti-p39 Ab were prepared by immunizing a rabbit with the highly purified p39 C3-cleaving proteinase. The specificity of this polyclonal anti-p39 Ab was analysed by immunoblotting techniques (Figure 3). While the preimmune serum of this rabbit did not react with p39 proteinase (lane 1), anti-p39 Ab reacted with purified p39 proteinase (lane 3) and not with human C3 (lane 2) used as a control.

This anti-p39 Ab was used on cell extracts in two sets of experiments. First, components solubilized from total membranes of DSm cells were incubated with anti-p39 Ab covalently linked to Protein A immobilized on Sepharose CL-4B. After

Table 2 Effect of proteinase inhibitors on C3-cleaving activity

Inhibitor	Specificity	Concn. (mM)	Inhibition of C3-cleaving activity (%)		
			Membrane extracts	Purified p39 proteinase	Conditioned medium
3,4-Dichloroisocoumarin	Serine proteinases	0.1	0	0	0
PMSF	Serine proteinases and some cysteine proteinases	1	0	0	0
1,10-Phenanthroline	Metalloproteinases and metal-activated proteinases	10	0	0	0
EDTA	Metalloproteinases and metal-activated proteinases	5	0	0	0
Pepstatin A	Some aspartic proteinases	0.01	10	0	0
E-64	Cysteine proteinases	0.1	100	100	100
		0.01	100	100	100
Iodoacetic acid	Cysteine proteinases and some serine proteinases	1	100	100	100
		0.1	64	60	58
Leupeptin	Some serine and cysteine proteinases	0.01	100	100	100

Figure 4 Effect of anti-p39 Ab on solubilized p39 proteinase

(a) Immunoabsorption of C3-cleaving activity from cell membrane extracts. ^{125}I -C3 was incubated with buffer (lane 1) or with membrane extracts of DSm melanoma cells, preincubated with IgG fraction of preimmune serum (lane 2) or anti-p39 Ab (lane 3) bound to Protein A-Sepharose. C3-cleaving activity was analysed by SDS/10%-PAGE under reducing conditions. (b) Inhibition of C3-cleaving activity by anti-p39 Ab. Total membrane extracts of DSm melanoma cells were incubated with purified IgG of anti-p39 Ab (●) or preimmune serum (○) at 4 °C for 60 min. Then solubilized components were tested for their C3-cleaving activity. The percentage inhibition was calculated by measuring the amount of C3dg fragment generated.

incubation, unbound components were incubated with ^{125}I -C3 and analysed for their proteinase activity (Figure 4a). The C3-cleaving activity was totally absorbed on immobilized anti-p39 Ab (lane 3), but not on preimmune serum Ig (lane 2). Secondly,

Figure 6 C3 cleavage by intact DSm cells

^{125}I -C3 was incubated with RPMI (lane 1) or with 10^6 DSm cells in RPMI (lane 2), for 60 min at 37 °C. Then cells were centrifuged and the supernatant was analysed by SDS/PAGE under reducing conditions.

membrane components solubilized from DSm were preincubated with various amounts of anti-p39 IgG for 60 min at 4 °C (Figure 4b). The C3-cleaving activity was then totally inhibited, while the immunoglobulins of preimmune serum had no effect. Thus anti-p39 Ab absorbs the p39 proteinase and directly inhibits C3-cleaving activity contained in crude membrane extracts.

Cell-surface expression

When DSm melanoma cells were incubated with antibodies,

Figure 5 Immunostaining of DSm melanoma cells with anti-p39 Ab

DSm cells were fixed on slides with paraformaldehyde, then incubated with an IgG fraction of anti-p39 Ab (a and b) or preimmune serum (c and d), followed by a rhodamine-conjugated anti-rabbit IgG. Photographs were taken without (a and c) or with (b and d) fluorescence filter.

Figure 7 Secretion of p39 proteinase by DSm melanoma cells

DSm cells were cultured in fetal-calf-serum-free culture media. At different incubation times, the conditioned media were collected, concentrated 12-fold and analysed. (a) Conditioned medium from DSm cells after 1 h (lane 1), 2 h (lane 2), 4 h (lane 3), 19 h (lane 4) and 24 h (lane 5) was concentrated 12-fold, then submitted to SDS/10%-PAGE under reducing conditions. Proteins were electrotransferred on to cellulose nitrate sheets, which were incubated with anti-p39 Ab followed by ^{125}I -labelled Protein A. (b) Concentrated conditioned media were tested for C3-cleaving activity. ^{125}I -C3 was incubated with conditioned media from DSm cells after 1 h (lane 1), 2 h (lane 2), 4 h (lane 3), 19 h (lane 4) and 24 h (lane 5). Then samples were submitted to SDS/10%-PAGE under reducing conditions.

anti-p39 IgG labelled the cell surface. In the control, immunoglobulin fractions of preimmune serum did not stain these cells (Figure 5).

In addition, intact cells incubated with ^{125}I -C3 cleaved C3 and generated the main C3dg fragment (Figure 6). Furthermore, preincubation of DSm cells with anti-p39 F(ab')_2 fragments increased up to 60 % the complement cell lysis initiated *in vitro* by anti-H2 monoclonal Ab and complement when compared with DSm cells preincubated with F(ab')_2 fragments of pre-immune serum immunoglobulins.

Secretion of p39 proteinase

DSm melanoma cells were incubated in fetal-calf serum-free culture medium for 1, 2, 4, 19 and 24 h, conditions in which cell viability was equal to 100 % (as determined by Trypan Blue exclusion). Conditioned media were concentrated then tested by immunoblotting techniques and for their C3-cleaving activity. As shown in Figure 7, the p39 protein and the C3-cleaving activity were detected in conditioned medium at 4 h, with a maximum reached at 24 h. This activity, like that of the purified p39 proteinase (Table 2), is of a cysteine-proteinase nature. Thus the p39 C3-cleaving activity was expressed on cell surface and was also secreted into the extracellular medium.

Amino acid sequences of the p39 C3-cleaving proteinase

Purified p39 C3-cleaving proteinase was submitted to a two-step amino acid analysis. The N-terminal sequence was determined for the first 17 amino acids and was:

Thr-Pro-Lys-Phe-Asp-Gln-Thr-Phe-Ser-Ala-Glu-Trp-His-Gln-Trp-Lys-Ser

Figure 8 Amino acid analysis of the p39 C3-cleaving proteinase

Purified p39 C3-cleaving proteinase was subjected to SDS/10%-PAGE under reducing conditions and electroblotted on to Immobilon P membrane. Internal regions of p39, proteolysed by endopeptidase Lys-C, were purified by reverse-phase chromatography on a DEAE-C18 HPLC column. Amino acid analysis of the N-terminus and three internal regions of the p39 proteinase were performed on an ABI 473 Sequenator (Applied Biosystems). These sequences show 100% identity with regions of mouse preprocathepsin L (EC 3.4.22.15). The arrow indicates where preprocathepsin L is processed into procathepsin L.

Then p39 was submitted to proteolysis by endopeptidase Lys-C. Generated p39 fragments were purified by reverse-phase chromatography on a DEAE-C18 HPLC column and peaks were isolated. The amino acid sequences of three of them were determined and were as follows:

peak 1: Tyr-Trp-Leu-Val-Lys

peak 2: Asn-Leu-Asp-His-Gly-Val-Leu-Leu-Val-Gly-Tyr-Gly-Tyr-Glu-Gly-Thr-Asp-Ser-Asn-Lys

peak 3: Asn-Ser-Trp-Gly-Ser-Glu-Trp-Gly-Met-Glu-Gly-Tyr-Ile-Lys

A homology search using the GenBank amino acid sequence data bank demonstrated that these four sequences are 100% identical with four regions of mouse preprocathepsin L (EC 3.4.22.15) (Figure 8). The N-terminal sequence of p39 corresponded to the region which started at position 18 of the preprocathepsin L sequence. The sequences of the peaks 1, 2 and 3 corresponded to amino acids 273–292, 295–299 and 300–313 respectively, localized in the C-terminal region of preprocathepsin L. Thus the p39 C3-cleaving proteinase carries amino-acid-sequence identity within four regions of procathepsin L.

DISCUSSION

In the present study we purified and identified a C3-cleaving proteinase highly expressed on DSm, a murine melanoma cell line. Preliminary analysis demonstrated that such C3-cleaving activity was not functionally related to either the p57 [10] or the p65 [11] C3-cleaving proteinases.

This new C3-cleaving activity was purified from membrane components solubilized in 10 mM CHAPS. This purification was achieved in four steps by submitting solubilized membrane proteins to chromatography on DEAE-Sepharose, Mono-Q, Mono-S and phenyl-Superose and monitoring the C3-cleaving activity by SDS/PAGE. This procedure resulted in a 1638-fold purification, with a 20% yield of the C3-cleaving activity present in cell membranes. The purified proteinase was characterized by SDS/PAGE, under reducing and non-reducing conditions, as a single chain of molecular mass 39 kDa; it was thus called the 'p39 C3-cleaving proteinase'.

The p39 C3-cleaving proteinase was identified as a cysteine proteinase, since its activity was inhibited by E-64, the specific cysteine-proteinase inhibitor but not by serine-, aspartic- nor metallo-proteinase inhibitors. In addition, the p39 cysteine proteinase was the only C3-cleaving proteinase present in solubilized DSm membranes, since: (1) specific proteinase inhibitors had an identical effect on the C3-cleaving activity present in total solubilized membrane components, secreted in conditioned medium or purified; (2) C3-cleaving activity present in total membrane extracts of DSm was fully absorbed on immobilized anti-p39 Ab prepared against highly purified p39 and was inhibited by anti-p39 Ab added to solubilized membrane components.

Determination of the amino acid sequence of the N-terminus and three other regions of the purified p39 cysteine proteinase demonstrated that this C3-cleaving proteinase carries 100% sequence identity within four regions of procathepsin L, suggesting that p39 may be a procathepsin L-like proteinase. However, different points should be taken into account.

Firstly, it was suggested by others, on the basis of protein and cDNA analysis, that murine cathepsin L was synthesized as a preproenzyme which is processed to a 39 kDa procathepsin L by removal of N-terminal 17 amino acids. Procathepsin L may be

further processed to two mature forms characterized by either a single 29 kDa polypeptide or a protein carrying two chains of 21 and 5 kDa linked by a disulphide bridge [21–23]. It was suggested that only these two (29 or 26 kDa) mature forms of procathepsin L carried enzymic activity, since an active-site-directed inhibitor did not react with the procathepsin L form [24] and the proregion of cathepsin L may inhibit its activity [25]. Conversion of murine procathepsin L in the lower-molecular-mass cathepsin L occurred only at pH values lower than 5.5 [26]. However, in our assays we did not detect any molecular-mass form lower than 39 kDa, even after incubating the purified p39 C3-cleaving proteinase at pH 7–7.5 or the proteinase present in conditioned medium for 24 h at 37 °C.

Secondly, the p39 cysteine proteinase cleaves C3 at pH 7–7.5, while it was described by others that purified cathepsin L cleaved synthetic substrates or proteins at pH values below 6 [26–28] and that commercial available cathepsin L cleaved C3 at pH 5 [29].

Thirdly, the p39 cysteine proteinase was mainly localized at the cell surface and was secreted in the extracellular medium, i.e. in conditions where pH is neutral and C3 accessible. Intact DSm cells cleaved labelled as well as unlabelled C3. Furthermore, preincubation of cells with F(ab')₂ fragments prepared from anti-p39 AB (which are able to inhibit the C3-cleaving activity of solubilized p39), increased up to 60% complement susceptibility of DSm cells, when compared with F(ab')₂ fragments prepared from preimmune Ab. Thus the results supported the notion that the p39 cysteine proteinase could regulate the properties of C3. While cathepsin L has been localized in lysosomes, the presence of cathepsin L-like activity which cleaves synthetic substrates has been suggested in plasma membranes of a murine melanoma cell line [30]. Confocal studies suggested association of cathepsin L with plasma membranes of human malignant colorectal tumour-cell line [31]. A cathepsin G which cleaves C3 has been described which is associated with plasma membranes of the monocytic cell line U937 [32]. However, cathepsin G has no common properties either with the p39 cysteine proteinase purified herein from melanoma cells or with cathepsin L: indeed, cathepsin G is a serine proteinase characterized by a molecular mass of 28 kDa and does not bear amino-acid-sequence identities with cathepsin L.

Thus full amino acid analysis of the p39 cysteine proteinase will be helpful to establish the exact relationship between this C3-cleaving proteinase and the cathepsin L proteinase family.

Our data constitute the first demonstration that a p39 cysteine proteinase that cleaves unlabelled as well as labelled C3 is expressed on, and is secreted from, a melanoma cell line. This p39 proteinase contributes to the protection of the cell against complement lysis by clearly diminishing its complement susceptibility. Thus, from all data presented herein, one may assume that the p39 C3-cleaving proteinase could play *in vivo* a biological role. Further studies are required to analyse the exact contribution of this new member of the C3-cleaving proteinase family in the resistance of melanoma cells to the immune system.

We thank Jacqueline Auville and Gérard Drevet for technical assistance. This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM), the Association pour la Recherche contre le Cancer (ARC), the Ligue Nationale Française contre le Cancer (LNFCC-Comité de Paris), Ministère de L'Éducation Nationale (DRED) and Fondation de France.

REFERENCES

- 1 Müller-Eberhard, H. J., and Schreiber, R. D. (1980) *Adv. Immunol.* **29**, 1–53
- 2 Harrison, R. A., and Lachman, P. J. (1980) *Mol. Immunol.* **17**, 9–20
- 3 Frade, R., Myones, B. L., Barel, M., Krikorian, L., Charriaud, C. and Ross, G. D. (1985) *Eur. J. Immunol.* **15**, 1192–1197
- 4 Hartmann, K. U., and Bokish, V. A. (1975) *J. Exp. Med.* **142**, 600–610

- 5 Hatzfeld, J., Charriaut-Marlangue, C., Levesque, J. P., Barel, M., Stancou, R., Krikorian, L., Hatzfeld, A. and Frade, R. (1987) *Ann. Inst. Pasteur/Immunol.* **138**, 451–455
- 6 Frade, R., Hermann, J. and Barel, M. (1992) *Biochem. Biophys. Res. Commun.* **188**, 833–842
- 7 Medof, M. E., Kinoshita, T. and Nussenweig, V. (1984) *J. Exp. Med.* **160**, 1558–1578
- 8 Liszewski, M. K., Tedja, I. and Atkinson, J. P. (1994) *J. Biol. Chem.* **269**, 10776–10779
- 9 Charriaut, C., Barel, M. and Frade, R. (1982) *Eur. J. Immunol.* **12**, 289–294
- 10 Charriaut-Marlangue, C., Barel, M. and Frade, R. (1986) *Biochem. Biophys. Res. Commun.* **140**, 1113–1120
- 11 Ollert, M. W., Frade, R., Fiandino, A., Panneerselvam, M., Petrella, E. C., Barel, M., Pangburn, M. K., Bredehorst, R., and Vogel, C. W. (1990) *J. Immunol.* **144**, 3862–3867
- 12 Hermann, J., Barel, M., and Frade, R. (1994) *Biochem. Biophys. Res. Commun.* **204**, 453–460
- 13 Lux, S. E., John, K. M. and Bennett, V. (1990) *Nature (London)* **344**, 36–42
- 14 Bennett, V. (1992) *J. Biol. Chem.* **267**, 8703–8706
- 15 Hammer, C. H., Wirtz, G. H., Renfer, L., Gersham, H. D. and Tack, B. F. (1981) *J. Biol. Chem.* **256**, 3995–4006
- 16 Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- 17 Laemmli U. K. (1970) *Nature (London)* **227**, 680–685
- 18 Markwell, M. A. K., and Fox, L. F. (1978) *Biochemistry* **17**, 4807–4817
- 19 Barel, M., Fiandino, A., Lyamani, F. and Frade, R. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 10054–10058
- 20 Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U. and Greaves, M. F. (1982) *J. Biol. Chem.* **257**, 10766–10769
- 21 Portnoy, D. A., Erickson, A. H., Kochan, J., Ravetch, J. V. and Unkeless, J. C. (1986) *J. Biol. Chem.* **261**, 14697–14703
- 22 Troen, B. R., Gal, S. and Gottesman, M. M. (1987) *Biochem. J.* **246**, 731–735
- 23 Joseph, L. J., Chang, L. C., Stamenkovich, D. and Sukhatme, V. P. (1988) *J. Clin. Invest.* **81**, 1621–1629
- 24 Mason, R. W., Wilcox, D., Wikstrom, P. and Shaw, E. N. (1989) *Biochem. J.* **257**, 125–129
- 25 Delaria, K., Fiorentino, L., Wallace, L., Tamburini, P., Brownell, E. and Muller, D. (1994) *J. Biol. Chem.* **269**, 25172–25177
- 26 Gal, S. and Gottesman, M. M. (1986) *J. Biol. Chem.* **261**, 1760–1765
- 27 Johnson, D. A., Barrett, A. J. and Mason, R. W. (1986) *J. Biol. Chem.* **261**, 14748–14751
- 28 Mason, R. W., Johnson, D. A., Barrett, A. J. and Chapman, H. A. (1986) *Biochem. J.* **233**, 925–927
- 29 Rey-Millet, C. A., Chesne, S. and Colomb, M. G. (1993) *Mol. Immun.* **10**, 855–864
- 30 Rozhin, J., Wade, R. L., Honn, K. V. and Sloane, B. F. (1989) *Biochem. Biophys. Res. Commun.* **164**, 556–561
- 31 Maciewicz, R. A., Wardale, R. J., Etherington, D. J. and Paraskeva, C. (1989) *Int. J. Cancer* **43**, 478–486
- 32 Maison, C. M., Villiers, C. L. (1991) *J. Immunol.* **147**, 921–926