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Immunology

Self-protection of cytotoxic lymphocytes: A soluble form of homologous restriction factor in cytoplasmic granules

(natural killer cells/T cells/inhibition of cytotoxicity/inhibition of the cytolytic lymphocyte protein)

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ABSTRACT **A soluble form of homologous restriction factor (HRF) has been isolated from the cytoplasmic granules of human large granular lymphocytes that were cultured in the presence of recombinant interleukin 2 for 2–3 weeks. The granule-derived protein (≈65 kDa) is soluble in detergent-free solution and reacts with antibody produced to membrane HRF. HRF was first described as a 65-kDa membrane protein of human erythrocytes capable of inhibiting the formation of transmembrane channels by the membrane attack complex of complement. It has also been isolated from activated human lymphocytes and shown to confer upon these cells relative resistance to lysis by the membrane attack complex and by the complement component C9-related protein of human cytotoxic lymphocytes. The soluble HRF of lymphocyte granules inhibits reactive lysis of erythrocytes by the membrane attack complex of human complement. It was also found to be a potent inhibitor of (*i*) the cytolytic activity of the C9-related protein of human cytotoxic lymphocytes, (*ii*) human large granular lymphocyte cytotoxicity, and (*iii*) the cytotoxic activity of human $CD8^+$ lymphocytes obtained by cell sorting from recombinant interleukin 2-activated peripheral blood mononuclear cells. It is proposed that granule-derived soluble HRF and cell surface-membrane-bound HRF are involved in the mechanism of self-protection of killer lymphocytes.**

Cytotoxic lymphocytes, although capable of killing a large variety of target cells, are relatively resistant to destruction by their own cytolytic activity (1–4). The molecular mechanism underlying the phenomenon of self-protection of cytotoxic lymphocytes is as yet unknown.

Homologous restriction factor (HRF) has been identified by this laboratory as a candidate for the molecular basis of lymphocyte self-protection activity (5). HRF was originally described as a membrane protein of normal human erythrocytes and leukocytes that interferes with the channel-forming function of the membrane attack complex (MAC) of complement (6). It has also been identified as the complement C8-binding protein of human erythrocytes (7). One of the functions of HRF is to protect blood cells from accidental lysis by the MAC of autologous complement as evidenced by the high susceptibility to MAC-mediated lysis of HRF-deficient human erythrocytes (8).

Two lines of evidence suggest that HRF may be involved in killer-cell self-protection. First, membrane HRF isolated from human erythrocytes and then bound to the surface of sheep erythrocytes inhibited antibody-dependent lysis of these cells by human large granular lymphocytes (LGLs) (9) and by the isolated cytolytic lymphocyte protein complement component C9-related protein (C9RP). C9RP derives its name from its relationship to the complement channel formers (10, 11) and is thought to be responsible for the cytolytic activity of human LGLs (10–12) and human cytotoxic T lymphocytes (13). Second, activation of peripheral human T lymphocytes led to expression of HRF on their surface. Concomitantly, these cells became relatively resistant to lysis by C9RP (5). This acquired resistance was abrogated by blocking lymphocyte surface HRF with antibodies to HRF, suggesting that resistance was due to lymphocyte membrane HRF.

The cytolytic C9RP has been located in the cytoplasmic granules of killer lymphocytes (10–12, 14, 15). When the effector cell and a recognized target cell make contact, the proteins contained in the cytoplasmic granules are secreted by exocytosis (16–18). The question arose, therefore, of whether HRF might also be located in the cytoplasmic granules to regulate C9RP activity. As will be shown below, a soluble form of HRF was isolated from cytoplasmic granules of human LGLs. This protein resembles membrane HRF with respect to molecular weight and reactivity with antibody made to membrane HRF. In addition, the soluble HRF is highly active in inhibiting reactive lysis by complement components C5b–9, C9RP-mediated lysis, and lymphocyte cytotoxicity.

MATERIALS AND METHODS

Human Peripheral Blood Mononuclear Cells (PBMCs). The cells were purified from heparin-treated normal blood by density-gradient centrifugation in lymphocyte-separation medium (Organon Teknika, Durham, NC). They were cultured in RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) containing recombinant interleukin 2 (10 units/ml) (Amgen Biologicals, Thousand Oaks, CA), 5% (vol/vol) heat-inactivated fetal bovine serum, and antibiotics (penicillin, streptomycin, and Fungizone). On day 6 of culture, the cells were stimulated with one dose of 0.2% phytohemagglutinin (M form, GIBCO), and they were harvested after 10–24 days. Starting with 2×10^8 PBMCs, a total of 2×10^9 cells usually were obtained by day 24.

Isolation of Cytoplasmic Granules. Granules from LGLs were harvested from 5×10^9 cells. The cells were pelleted, washed twice with Hanks' balanced salt solution, resuspended in 20 ml of relaxation buffer containing 100 mM KCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM Pipes (pH 6.8), 1.25 mM EGTA (14, 15), and subjected to N_2 cavitation (19). Nuclei were removed by centrifugation at 1000 rpm for 10 min in a Sorvall H1000B rotor. The supernatant was applied to a Percoll gradient as described (19). By using Percoll density gradient markers (Pharmacia Fine Chemicals, Piscataway, NJ) as standards, the fractions from 1.076 to 1.41 g/ml were

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Abbreviations: HRF, homologous restriction factor; HRF-G, cytoplasmic granule-derived HRF; HRF-M, membrane-derived HRF; MAC, membrane attack complex of complement; LGL, large granular lymphocytes; C9RP, C9-related protein; PBMC, peripheral blood mononuclear cell.

pooled. Percoll was removed from the granule preparation by centrifugation in an SW 41 Beckman rotor (Beckman) at 35,000 rpm for 16 hr at 4°C. The granules were removed as a visible band above the hard Percoll pellet.

Preparation of Soluble Granular Proteins. Whole granules were lysed in 2 M NaCl containing 20 mM Tris·HCl (pH 7.4), 10 mM EDTA, 10 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM benzamidine for 6 hr at 4°C and subjected to repeated freeze-thawing. Insoluble material was removed by centrifugation for 20 min at 13,400 × g. The supernatant was dialyzed against starting buffer for column chromatography containing 0.1 M NaCl, 20 mM Tris·HCl (pH 7.4), and 5 mM EDTA.

Purification of Cytoplasmic Granule-Derived HRF (HRF-G). Two methods were used: anti-HRF affinity chromatography and Mono Q anion-exchange chromatography. In the latter case, 250 μg of soluble granular protein was applied to a Mono Q (HR5/5) column on an FPLC system (Pharmacia). The column was equilibrated with 1.75 mM NaCl in 20 mM Tris·HCl (pH 8) and the protein was eluted initially with the same solvent (13 ml). A NaCl concentration gradient from 1.75 to 245 mM (17 ml) was used to elute the adsorbed proteins. HRF was identified by its ability to inhibit C5b–9-mediated lysis of sheep erythrocytes.

For isolation by affinity chromatography, a 10-ml column of Sepharose 4B with 100 mg of bound anti-HRF immunoglobulin was used. The antibody was raised in rabbits immunized with isolated human erythrocyte membrane-derived HRF (HRF-M) (9). The column was equilibrated with starting buffer (0.1 M NaCl/5 mM EDTA/20 mM Tris·HCl, pH 7.4). About 700 μg of soluble granular protein was applied to the column, and the column was extensively washed with starting buffer. The adsorbed protein was eluted first with 3 M NaCl and then with 4 M KBr. The eluates were dialyzed against starting buffer and concentrated to 1 ml. The total yield of HRF-G was 10 to 15 μg.

Isolation of C9RP. C9RP was isolated by affinity chromatography with a rabbit anti-C9RP-Sepharose immunoadsorbent column (11, 13) from granules of human PBMCs, maintained in culture for 20 days in rIL-2 (10 units/ml).

Gel Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was performed with an acrylamide gradient of 3–18%. Immunoblots were carried out with the reagents and by the instructions provided by Bio-Rad. The antiserum was made in rabbits by immunization with HRF isolated from human erythrocyte membranes (6, 9).

Reactive Lysis by C5b–9. Chicken erythrocytes (10^7 cells) were exposed to the isolated complement components C5b,6, C7, C8, and C9 in molar ratios of 1:1:1:10. The amount of C5b–9 was selected to produce 50% lysis in 15 min at 37°C (9). In HRF inhibition experiments, various amounts of HRF-G were added to cell suspension 10 min prior to the addition of C5b–9.

Cytotoxicity Assay. M21 human melanoma cells or human K562 erythroleukemia cells were used as target cells. Cytotoxic activity of LGLs or $CD8^+$ lymphocytes was determined in a 4-hr ^{51}Cr-release assay. Target cells (4×10^6 cells per 0.5 ml) were labeled with ^{51}Cr (200 μCi per 0.5 ml; 1 Ci = 37 GBq) for 2 hr at 37°C and washed three times. They were resuspended at 4×10^6 cells per ml. The effector cells were titrated into wells of a 96-well microtiter plate, and 10^5 target cells were added to each well. In HRF inhibition experiments, HRF-G was added to the effector cells before addition of the target cells. The effector/target cell ratio was between 0.8:1 and 6.7:1. Each experimental point in the killing assays represents the average of duplicate determinations.

In experiments in which inhibition of C9RP-mediated lysis was tested, the sequence of addition was C9RP, HRF-G, target cells, and $CaCl_2$ (10 mM, final concentration). When $CD8^+$ cells were employed as effector cells, PBMCs were stimulated with rIL-2 (100 units/ml) for 3 days. Fluorescein isothiocyanate-conjugated OKT8 (anti-CD8); Ortho Diagnostics, was used to stain the $CD8^+$ cells for cell sorting, which was performed by a FACSTAR (Becton Dickinson).

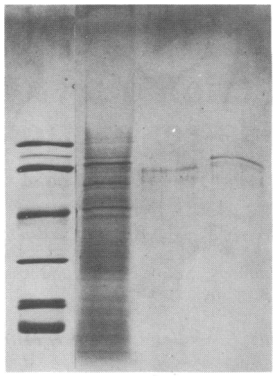

FIG. 1. Soluble HRF and C9RP isolated from human LGL cytoplasmic granules (gran. cont.) and analyzed by $NaDodSO_4$/polyacrylamide (3–18%) gradient gel electrophoresis. The soluble proteins of isolated granules were separated by Mono Q anion-exchange chromatography with a NaCl concentration gradient. HRF was eluted at 115 mM NaCl and C9RP at 213 mM NaCl. The gel was stained with Coomassie blue. Molecular weight standards (MW stnds) were phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme.

S-protein. S-protein was purchased from Calbiochem-Behring (La Jolla, CA). The material had the characteristic properties of S-protein and was active in inhibiting reactive lysis of erythrocytes by C5b–9 (20).

RESULTS

Isolation of Soluble HRF from Granules of LGLs. Human LGLs were purified from peripheral blood and cultured in the presence of recombinant interleukin 2 for 2–3 weeks. Approximately 5×10^9 cells were subjected to N_2 cavitation to release the cytoplasmic granules that were then purified by

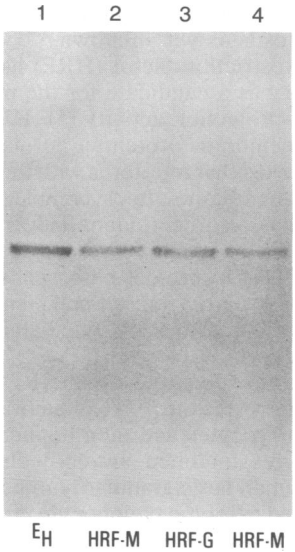

FIG. 2. Comparison of HRF-G and HRF-M by immunoblotting. The acrylamide gradient was 3–18%, and the blot was developed with anti-HRF-M. HRF of solubilized human erythrocyte membranes (lane 1, E_H) is also shown. The molecular weight of HRF-G (lane 3) appears to be slightly lower than that of HRF-M (65,000) (lanes 2 and 4).

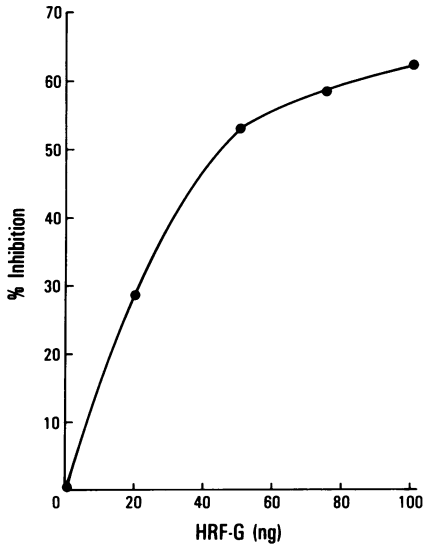

FIG. 3. HRF-G inhibition of reactive lysis of chicken erythrocytes by C5b–9. Cells (10^7 cells) were exposed to the concentrations of C5b,6, C7, C8, and C9 that caused 50% lysis in 15 min at 37°C. The indicated amounts of HRF-G were added to the cell suspension prior to addition of C5b–9, and cell lysis was measured.

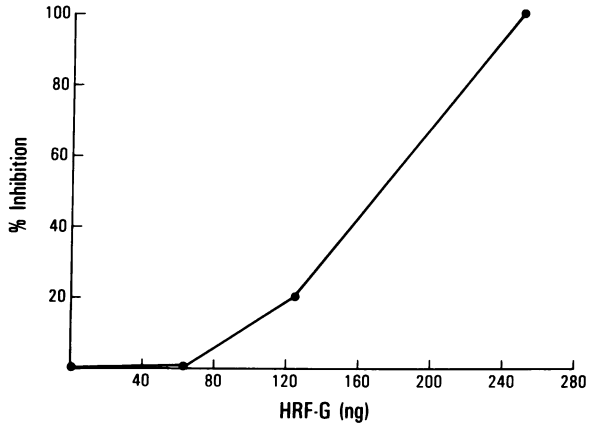

FIG. 4. Inhibition of C9RP-mediated lysis of M21 human melanoma cells by HRF-G. ^{51}Cr-labeled cells (10^5 cells) were incubated with ≈2 μg of C9RP and the indicated amounts of HRF-G in a total volume of 200 μl containing 10 mM $CaCl_2$ for 4 hr. In absence of HRF-G, cell lysis was 63%.

Percoll gradient centrifugation. About 1 mg of total granule protein was thus obtained, and the granules were lysed by sonication and freeze-thawing in 2 M NaCl. After removal of insoluble material, the soluble granule content was dialyzed against 0.1 M NaCl and subjected to affinity chromatography on anti-HRF bound to Sepharose. Alternatively, the soluble proteins from the granules were separated by anion-exchange chromatography with a Mono Q column. No detergents were present during these procedures. An ≈65-kDa protein subsequently identified as HRF was eluted at 115 mM NaCl and the 70-kDa C9RP was eluted at 213 mM NaCl. Fig. 1 depicts the $NaDodSO_4$/PAGE pattern of the chromatographically purified soluble HRF-G and C9RP. Two preparations of soluble HRF-G were obtained by anion-exchange chromatography, and three preparations were obtained by anti-HRF immunoadsorbent chromatography. Approximately 20 μg of soluble HRF-G could be obtained from 1 mg of granule protein.

Relationship of HRF-G to HRF-M. Comparative immunoblots of HRF-G and HRF-M showed similar reactivity with antibody made to HRF-M (Fig. 2). The apparent molecular weight of HRF-G was slightly lower than that of HRF-M, which is ≈65,000.

The activity of HRF-G was tested with respect to its ability to inhibit reactive lysis of chicken erythrocytes by C5b–9. The amount of C5b–9 used caused 50% lysis of 10^7 cells in 15 min at 37°C. The dose–response curve shown in Fig. 3 indicates a dose-dependent inhibition of C5b–9 activity, with ≈100 ng of HRF-G causing 63% inhibition of the lysis. At this level of inhibition the molar ratio of HRF-G/C5b–9 was ≈1:2. HRF-G has no demonstrable affinity for the erythrocytes, but rather exerted its activity from the fluid phase.

Inhibition of C9RP-Mediated Cell Lysis by HRF-G. M21 human melanoma cells (10^5 cells) were incubated with isolated C9RP for 4 hr. Approximately 2 μg of C9RP caused 62% killing. This reaction was inhibited 50% by ≈175 ng of HRF-G and was completely suppressed by ≈250 ng of HRF-G (Fig. 4). S-protein, used as a control, had no effect on this reaction (data not shown).

Inhibition of Natural Killer Cell Cytotoxicity by HRF-G. M21 cells (10^5 cells) were lysed to a level of 97% by human LGLs at an effector/target cell ratio of 5.5:1. Fifty percent inhibition of lysis was achieved with 72 ng of HRF-G per 200 μl of reaction mixture and 98% inhibition was achieved with 140 ng. S-protein was used as control, which did not affect the cytotoxic reaction, even at >10 μg per 200 μl (Fig. 5).

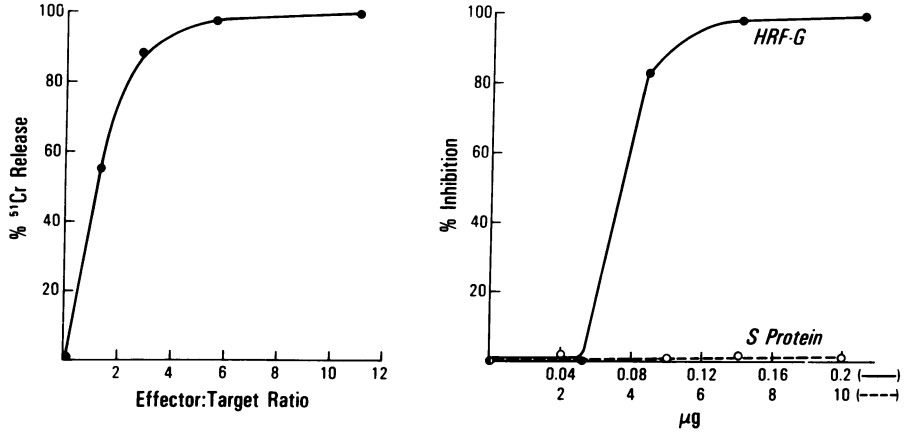

FIG. 5. Inhibition of LGL cytotoxicity by HRF-G. ^{51}Cr-labeled M21 melanoma cells (10^5 cells) were incubated with human LGLs at an effector/target cell ratio of 5.5:1 in the presence of the indicated amounts of HRF-G in a total volume of 200 μl for 4 hr. In absence of HRF-G, cell lysis was 97%. S-protein was without effect.

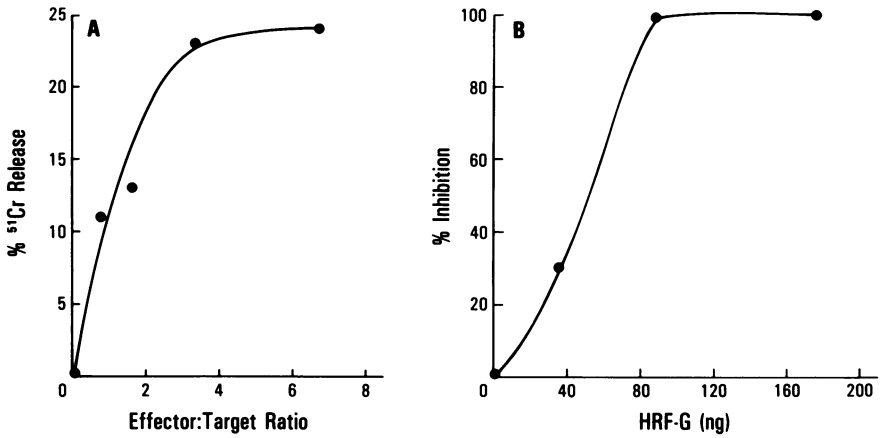

FIG. 6. Inhibition of CD8$^+$ lymphocyte cytotoxicity by HRF-G. ^{51}Cr-labeled K562 human erythroleukemia cells (10^5 cells) were incubated with peripheral blood activated cytotoxic T lymphocytes at an effector/target cell ratio of 3.3:1 in the presence of the indicated amounts of HRF-G in a total volume of 200 μl for 4 hr. In the absence of HRF-G, cell lysis was 25%. The cytotoxic T lymphocytes were obtained by fluorescence-activated cell sorting from human PBMCs activated for 3 days with recombinant interleukin 2 (100 units/ml).

Inhibition of CD8$^+$ Cell Cytotoxicity by HRF-G. Human peripheral blood mononuclear cells were activated for 3 days with recombinant interleukin 2 (100 units/ml). The CD8$^+$ lymphocytes were subsequently obtained by fluorescence-activated cell sorting. These cells killed ≈25% of 10^5 K562 cells at an effector/target cell ratio of 3.3:1. Fifty percent inhibition of this reaction was observed in ≈50 ng of HRF-G, and 100% inhibition was seen with ≈88 ng (Fig. 6).

DISCUSSION

The experimental developments that preceded and prompted the present study are as follows: (*i*) Membrane HRF was isolated originally by virtue of its affinity for human C9 (6). (*ii*) Isolated HRF-M was shown to inhibit transmembrane channel formation by the MAC, specifically by C8 and C9 (6, 7). (*iii*) Isolated HRF-M inserted into sheep erythrocyte membranes was shown to inhibit the lytic action of lymphocyte C9RP and to inhibit antibody-dependent lymphocyte cytotoxicity (9). Considering that cytotoxic lymphocytes use C9RP as a tool to lyse target cells, HRF-M appeared an obvious candidate for fulfilling the function of self-protection. Further exploration revealed (*i*) that when resting T cells acquired cytotoxicity, which correlates with C9RP synthesis (21, 22), they increased the expression of cell surface HRF, (*ii*) that activation of cytotoxic T lymphocytes conferred relative resistance to C9RP lysis upon these cells, and (*iii*) that this acquired resistance was abrogated by anti-HRF (5).

Whereas these results suggested that HRF-M may be involved in the mechanism of self-protection for killing lymphocytes, they also raised the possibility that HRF is contained in the cytoplasmic granules that harbor the cytolytic C9RP. However, the protein that was isolated from the granules of LGLs by anti-HRF-M-immunoadsorption differs from HRF-M in that it is soluble in detergent-free solution and does not bind firmly to cells, such as erythrocytes. Although HRF-G appears to lack the membrane-seeking anchor of HRF-M, its molecular weight on NaDodSO$_4$/PAGE is close to that of HRF-M. Since HRF-M probably belongs to the group of membrane proteins possessing a phosphatidylinositol anchor (8), it is conceivable that HRF-G constitutes the phosphoglycolipid-free form of HRF-M.

HRF-G efficiently inhibits reactive lysis of erythrocytes by C5b–9. Unlike HRF-M, which exhibits this activity when it is bound to liposomes or erythrocytes (6, 8, 9), the soluble HRF-G exerts this effect from the fluid phase. In this regard it resembles S-protein, the primary MAC inhibitor of serum (20). S-protein binds to the metastable membrane binding site of nascent C5b–7 and allows binding of C8 and C9 but prevents C9 polymerization (23). It thus causes the forming C5b–9 complex to remain in the fluid phase instead of binding to the target membrane. The molecular mode of interference of HRF-G with C5b–9 assembly is unknown. HRF-G differs from S-protein in immunochemical properties (unpublished observation) and with respect to its effect on cellular cytotoxicity.

The strong inhibition of C9RP-mediated lysis of M21 melanoma cells by HRF-G may be interpreted as evidence for direct physical interaction between HRF-G and C9RP. Whether this interaction occurs with native or with unfolding and polymerizing C9RP remains to be determined. Preliminary evidence was obtained for the binding of radiolabeled HRF-G to C9RP chemically linked to particles (unpublished observation).

The inhibition of LGL and CD8$^+$-cell cytotoxicity by HRF-G suggests that in both systems C9RP was an essential mediator. S-protein used as a control in the LGL cytotoxicity experiments was without effect. That C9RP plays a role in lymphocyte cytotoxicity is also borne out by the strong inhibitory effects observed with certain monoclonal antibodies to C9RP in experiments with either human LGL or CD8$^+$ lymphocytes as effector cells (24).

The reported work shows that the soluble HRF-G can function as a potent inhibitor of lymphocyte cytotoxicity and of C9RP cytolytic activity *in vitro*. This activity of HRF-G as well as the functional properties of lymphocyte HRF-M (5) are entirely consistent with the hypothesis that HRF may confer self-protection on killer lymphocytes. HRF-M may be envisioned to protect the surface of a killer lymphocyte, and HRF-G may control C9RP within the granules and during exocytosis. In this case, a mechanism must be postulated that is capable of separating HRF-G from C9RP outside the lymphocyte, thereby allowing C9RP to act on the target membrane.

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