EDTA, leupeptin (5 μg/ml), and 2 mM phenylmethylsulfonyl fluoride]. A portion of the homogenate was diluted to 0.6 ml and adjusted to 1.4% SDS before measurement of the protein concentration [O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951)]. The homogenate was then diluted with homogenization buffer to a protein concentration of ~1.75 mg/ml. One part deoxycholate solution (0.56%, pH 7.5) was added to 10 parts diluted homogenate while mixing. Each sample was incubated at 4°C for 30 min and then centrifuged at 103,000*g* for 60 min. The supernatant was removed, diluted 1:1 with 15/40 buffer [15 mm tris (pH 7.4), 40 mM NaCl, 1 mM EDTA, and 0.02% NaN_3], and then dialyzed overnight against 15/40 buffer at 4°C. Portions of the dialyzed supernatant were assayed for TG transfer activity, and immunoblot analysis was performed to detect the 88-kD MTP subunit. TG transfer activity was measured as the protein-stimulated rate of TG transfer from donor small unilamellar vesicles (SUVs) to acceptor SUVs. Vesicles of the desired composition were prepared by bath sonication in 15/40 buffer as described previously (*5*). The donor and acceptor PC was labeled by adding traces of [^{3}H]dipalmitoyl PC {phosphatidylcholine L-alpha-dipalmitoyl-[2-palmitoyl-9,10,^{3}H(*N*)]; 33 Ci/mmol; DuPont Biotechnology Systems} to a specific activity of ~100 cpm/nmol. Donor vesicles containing 40 nmol of egg PC, 0.2 mole percent [^{14}C]triolein {triolein [carboxyl-^{14}C]; ~110 Ci/mol; DuPont Biotechnology Systems}, and 7.3 mole percent bovine heart cardiolipin (Sigma) were mixed with acceptor vesicles containing 240 nmol of egg PC and 0.2 mol percent unlabeled TG, 5 mg of fatty acid–free bovine serum albumin, and a portion of the MTP samples in 0.9 ml of 15/40 buffer, and the mixture was incubated for 1 hour at 37°C. The transfer reaction was terminated by the addition of 0.5 ml of a DEAE-cellulose suspension (*5*) and low-speed centrifugation to sediment selectively the donor vesicles containing the negatively charged cardiolipin. The measured amounts of [^{14}C]TG (transferred from donor to acceptor SUVs) and [^{3}H]PC (marker of acceptor SUV recovery) were used to calculate the percentage TG transfer from donor to acceptor SUVs. First-order kinetics were used to calculate the total TG transfer (*5*). To calculate the protein-stimulated rate of TG transfer, the rate of TG transfer in the absence of transfer protein was subtracted from that in the presence of MTP. To confirm that TG hydrolysis was not interfering with our ability to measure lipid transfer, after the assay of two subjects we extracted the acceptor vesicle lipid (which contained the transported lipid) and confirmed the identity of the TG by thin-layer chromatography. All the ^{14}C had a mobility identical to that of authentic TG, confirming that intact TG was transported in the assays. In addition, the human MTP was characterized for its heat stability. MTP was inactivated when heated to 60°C for 5 min. The loss of activity demonstrates that transfer activity attributable to an intracellular form of the cholesteryl ester transfer protein, which is stable at 60°C [J. Ihm, J. L. Ellsworth, B. Chataing, J. A. K. Harmony, *J. Biol. Chem.* **257**, 4818 (1982)], was not being measured.

11. The clinical description and other relevant information for patient M.K. with Anderson's disease are presented elsewhere [F. Lacaille *et al.*, *Arch. Fr. Pediatr.* **46**, 491 (1989); M.-E. Bouma, I. Beucler, L. P. Aggerbeck, R. Infante, J. Schmitz, *J. Clin. Invest.* **78**, 398 (1986)].
12. The clinical description and relevant data for patient C.D. with homozygous hypobetalipoproteinemia are presented elsewhere [G. Gay *et al.*, *Rev. Med. Interne* **11**, 273 (1990); J.-Y. Scoazec *et al.*, *Gut* **33**, 414 (1992)].
13. To identify the 88-kD component of MTP in tissue homogenates, we fractionated aliquots of proteins to be tested by SDS–polyacrylamide gel electrophoresis and then transferred the separated proteins to nitrocellulose with a Bio-Rad Trans-blot cell. After incubation with a nonfat milk solution, the nitrocellulose filter was incubated overnight at room temperature with an aliquot of antiserum to the 88-kD protein (1:300 dilution) or affinity-purified antibodies (1:25 dilution). Immunoreactive proteins were visualized with horseradish peroxidase–coupled goat antibodies to rabbit immunoglobulin G (Bio-Rad) and a standard developing solution.
14. The production and characterization of the antiserum to the 88-kD protein have been previously described (*7*). The antiserum immunoprecipitates MTP protein and activity, but direct inhibition of MTP activity has not been demonstrated. Affinity-purified antibodies were prepared as follows: Purified MTP (8 to 10 mg) was coupled to 4 ml of Bio-Rad Affigel 15. Antibodies were partially purified from the antiserum by $(NH_4)_2SO_4$ precipitation [226 mg of $(NH_4)_2SO_4$ per milliliter of serum]. After centrifugation, the pellet was suspended and applied to the MTP-affigel. The column was washed with 100 ml of 10 mM tris (pH 7.5), followed by 100 ml of 10 mM tris (pH 7.5) containing 500 mM NaCl. Antibodies were eluted with 50 ml of 100 mM glycine (pH 2.5) into 5 ml of 1 M tris (pH 8.0).
15. With unfractionated antiserum, the 88-kD band of MTP was detectable in the soluble proteins released from <3 μg of intestinal homogenate protein of three of four control subjects investigated. In the fourth subject, who had the lowest level of TG transfer activity of the controls, the 88-kD component of MTP was detectable in the soluble proteins from 10 μg of intestinal homogenate protein.
16. J. R. Wetterau *et al.*, unpublished data.
17. J. A. Higgins and J. L. Hutson, *J. Lipid Res.* **25**, 1295 (1984).
18. D. R. Janero and M. D. Lane, *J. Biol. Chem.* **258**, 14496 (1983).
19. K. Boström *et al.*, *ibid.* **263**, 4434 (1988).
20. K. J. Lackner *et al.*, *J. Clin. Invest.* **78**, 1707 (1986).
21. We thank F. Maurer Chagrin (Hôpital Bichat, Paris, France) for providing biopsy samples from normal subjects, N. Verthier for technical assistance, and the University of Cincinnati for the antiserum to the 88-kD component of MTP. Supported by the Bristol-Myers Squibb Pharmaceutical Research Institute, the Centre National de la Recherche Scientifique, the Institut National de la Santé et Recherche Médicale (grant CRE 910301 and a France–U.S. cooperation grant), A.R.C.O.L. (the Committee for the Coordination of Research on Cholesterol in France), and NIH grant HL18577 (to L.P.A.).

26 May 1992; accepted 25 August 1992

Tyrosine Phosphorylation of CD22 During B Cell Activation

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Ligation of the antigen receptor on B cells induces the rapid phosphorylation of tyrosine on a number of cellular proteins. A monoclonal antibody that recognized a tyrosine-phosphorylated cell surface protein that was present in activated B cells was generated. Amino acid sequence analysis showed that this 140-kilodalton protein was CD22, a B cell–specific cell surface glycoprotein and putative extracellular ligand of the protein tyrosine phosphatase CD45. Tyrosine phosphorylation of CD22 may be important in B cell signal transduction, possibly through regulation of the adhesiveness of activated B cells.

The B lymphocyte antigen receptor complex consists of membrane immunoglobulin (Ig), at least two accessory molecules (Ig-α and Ig-β) (*1*), several members of the Src family of protein tyrosine kinases (*2*, *3*), and a 72-kD protein tyrosine kinase that may be encoded by the *syk* gene (*4*, *5*). Cross-linking of surface Ig induces rapid increases in both tyrosine protein phosphorylation (*6–8*) and inositol phospholipid hydrolysis (*9*). Evidence suggests that the increased inositol phospholipid hydrolysis is induced, at least in part, by tyrosine phosphorylation. (i) Phospholipase C-γ, which is regulated by tyrosine phosphorylation in fibroblasts (*10*), is phosphorylated on tyrosine during B cell activation (*11*). (ii) The increase in free intracellular Ca^{2+} that results from inositol phospholipid hydrolysis is prevented by treatment of B cells with herbimycin, an inhibitor of tyrosine protein phosphorylation (*12*). (iii) Expression of the protein tyrosine phosphatase CD45 is required for the stimulation of phosphatidylinositol hydrolysis in a murine plasmacytoma (*13*).

Protein tyrosine phosphorylation may in fact represent the trigger or initial intracellular biochemical signaling event induced by the ligation of surface Ig. It is not clear how ligation of this receptor complex induces increased substrate phosphorylation, but it is likely that the Src-family kinases or the 72-kD kinase plays a role.

Phospholipase C-γ is not the only protein to undergo rapid tyrosine phosphorylation after cross-linking of surface Ig with antibody. Approximately ten newly phosphorylated proteins can be detected by immunoblotting of total cell lysates with antibodies to phosphotyrosine (*6–8*), including the *vav* proto-oncogene product (*14*), the 72-kD cytosolic protein tyrosine kinase (*4*, *6*), the 42-kD mitogen-activated (MAP)/

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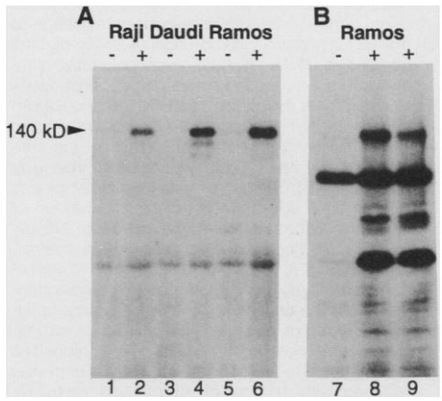

Fig. 1. Analysis of tyrosine phosphorylation of p140, which is recognized by MARA 2. (**A**) As indicated at the top of each lane, cells were stimulated (indicated by a plus sign) with anti-Ig (20 μg/ml) for 2.5 min at 37°C (*17*) or left unstimulated (indicated by a minus sign). Cells (1.5×10^6) were lysed in RIPA buffer, and precipitation was carried out with MARA 2 antibody. Samples were analyzed by SDS-PAGE and protein immunoblotting with antiphosphotyrosine antibodies and ^{125}I-labeled protein A (*30*). The cell lines used are shown above each lane. The 140-kD band is indicated by the arrow. (**B**) Ramos cells were left unstimulated or stimulated with anti-Ig and lysed in RIPA buffer at 10^7 cells/ml. The lysate was depleted of proteins recognized by MARA 2 by four consecutive immunoprecipitations (lane 9); 3.5×10^5 cell equivalents per lane were analyzed by antiphosphotyrosine protein immunoblotting as described in (A).

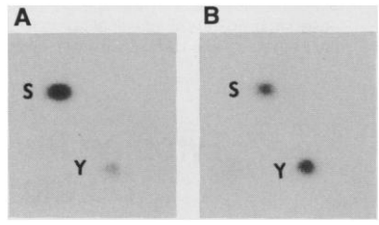

Fig. 2. Phosphoamino acid analysis of p140. Ramos cells labeled biosynthetically with $^{32}P_i$ were left unstimulated (**A**) or were stimulated with anti-Ig (**B**), lysed in RIPA buffer, and immunoprecipitated with MARA 2. The immunoprecipitated proteins were subjected to SDS-PAGE and transferred to Immobilon-P (Millipore). After detection by fluorography, the p140 band was excised and subjected to phosphoamino acid analysis (*31*). Phosphoamino acids were detected by fluorography with an intensifying screen (5-day exposure). Individual phosphoamino acids were identified by alignment with internal standards. S, phosphoserine, and Y, phosphotyrosine.

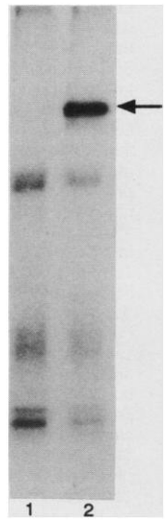

Fig. 3. Analysis of p140 expression on the cell surface. Ramos cells were labeled by lactoperoxidase-catalyzed iodination with ^{125}I, lysed in RIPA buffer, and subjected to immunoprecipitation with either tissue culture medium alone (lane 1) or MARA 2 tissue culture supernatant (lane 2). Immunoprecipitates from 5×10^6 cell equivalents were analyzed by SDS-PAGE, and precipitated proteins were detected by fluorography with an intensifying screen. The 140-kD band is indicated by the arrow.

extracellular signal–regulated (ERK) kinase (*15*), Ig-α and Ig-β (*8*, *16*), and possibly one or more Src-like kinases (*2*).

To characterize the unidentified proteins that undergo tyrosine phosphorylation during B cell activation, we purified tyrosine-phosphorylated proteins from the Ramos line of human Burkitt's lymphoma B cells that had been activated with antibodies to immunoglobulin (anti-Ig) and prepared monoclonal antibodies reactive to the phosphorylated proteins (*17*). One antibody, MARA 2, recognized a 140-kD tyrosine-phosphorylated protein, p140. The tyrosine phosphorylation of p140, which was detectable in unstimulated cells, increased approximately fivefold after exposure of Daudi, Raji, and Ramos human Burkitt's lymphoma cell lines (Fig. 1A) or human tonsillar B cells (*18*) to anti-Ig, as determined by immunoblotting with antibodies to phosphotyrosine (antiphosphotyrosine). Cross-linking of major histocompatibility complex (MHC) class I molecules did not induce tyrosine phosphorylation of p140 (*18*). The 140-kD protein was detectable when total lysates of anti-Ig–stimulated Ramos cells were analyzed by immunoblotting with antiphosphotyrosine. Scanning densitometry showed that repeated precipitation with MARA 2 removed approximately 50% of the 140-kD band seen in the cell lysates (Fig. 1B). Phospholipase C-γ1 and phospholipase C-γ2 have electrophoretic mobilities similar to that of p140 and make up some of the tyrosine-phosphorylated material migrating with this mobility.

Phosphoamino acid analysis of p140 labeled biosynthetically with ^{32}P-labeled inorganic phosphate $^{32}P_i$ verified the presence of phosphotyrosine. Phosphotyrosine represented ~10% of the phosphoamino acids in p140 in untreated cells and 40 to 50% of the phosphoamino acids after stimulation of the cells with anti-Ig (Fig. 2).

To determine the fraction of p140 molecules that underwent tyrosine phosphorylation, we estimated by biosynthetic labeling with $^{32}P_i$ the stoichiometry of phosphorylation of the protein after stimulation of cells with anti-Ig (*19*). The protein was found to contain ~1.5 mol of phosphate per mole of protein. Chymotryptic peptide mapping suggests that anti-Ig stimulation induces the phosphorylation of one major peptide and two minor peptides. Because phosphotyrosine constituted approximately half of the phosphoamino acid content of the protein from stimulated cells, we estimate that approximately half to three-quarters of the p140 molecules are phosphorylated on tyrosine after stimulation with anti-Ig.

Biosynthetic labeling and immunoprecipitation with MARA 2 resulted in the detection of p140 in four human B cell lines. In contrast, the protein could not be detected in a number of other human cell lines: HeLa cells, two T cell lines (HPB-MLT and HSB-2), an erythroleukemia cell line (HEL), a neuronal cell line (HTB), a kidney cell line (293), and a myeloid cell line (U-937) (*18*).

The 140-kD protein was labeled efficiently by lactoperoxidase-catalyzed iodination of intact cells (Fig. 3), which indicates that it is expressed at the cell surface. Because the binding of MARA 2 to intact cells could not be detected by immunofluorescence (*18*), we suspect that the antibody recognizes either an intracellular determinant or a determinant that is cryptic before cell lysis.

To identify p140, we purified it by immunoprecipitation and gel electrophoresis, transferred it to a polyvinylidene difluoride membrane, digested it with trypsin, and performed automated sequence analysis of several tryptic peptides (Fig. 4A). The sequence of the peptide eluted at 34 min was identical to that of residues 206 to 215 of the human CD22 protein (*20*, *21*). Additionally, the peak eluted at 51 min was deduced to be a mixture of two peptides comprising residues 454 to 465 and 538 to 545 of human CD22. Because CD22 is a 140-kD cell surface protein whose expression is restricted to B cells, we concluded that the tyrosine-phosphorylated protein that we had identified with the MARA 2 antibody was CD22. This conclusion was supported by the finding that precipitation of CD22 with a known monoclonal antibody to CD22 showed that it underwent increased tyrosine phosphorylation after stimulation of Ramos cells with anti-Ig (Fig. 4B).

It is possible that the phosphorylation of CD22 on tyrosine plays a role in B cell activation. Expression of CD22 is limited to a subset of both peripheral blood and tonsillar B lymphocytes (*22*). In the tonsil, cell-surface expression of CD22 is most prominent on recirculating surface Ig^+ small B cells in primary follicles and in the follicular mantle zone of the germinal cen-

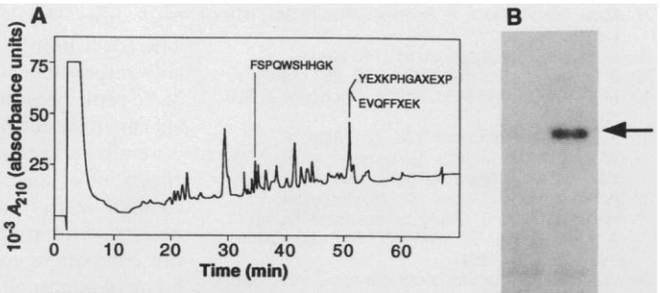

Fig. 4. (**A**) High-performance liquid chromatography profile of a tryptic digest of p140. The p140 was isolated from a lysate of 2.2 × 10^9 Ramos cells by immunoprecipitation with antibodies from 125 ml of MARA 2 hybridoma tissue culture supernatant. The precipitate was dissolved in 750 μl of SDS-PAGE sample buffer and resolved on a 1-mm-thick SDS–polyacrylamide gel. Protein was electrophoretically transferred to Immobilon-P in 10 mM CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] at pH 11 (Sigma) and 10% methanol. Protein bands were identified with amido black and excised. After digestion with trypsin (*32*), the sample was fractionated on a Vydac C_{18} column (2.1 by 150 mm, 300 Å pore size, and 5-μm particle size). After isocratic elution at 100% solvent A (0.05% trifluoroacetic acid in water) for 5 min, a gradient to 45% solvent B (90% acetonitrile, 10% water, and 0.050% trifluoroacetic acid) was applied within 60 min. The flow rate was 0.2 ml/min. Fractions were collected manually on the basis of absorbance at 210 nm (A_{210}). Sequence analysis of eluted peptides was done on a protein sequencer (Model 470A; Applied Biosystems). The assignment of the two sequences in fraction 58 was based on comparison of the crude data to the published sequence of CD22 (*20*). Peptide sequences are shown in the single-letter code: A, Ala; E, Glu; F, Phe; G, Gly; H, His; K, Lys; P, Pro; Q, Gln; S, Ser; V, Val; W, Trp; X, ambiguous; and Y, Tyr. (**B**) Analysis of tyrosine phosphorylation of CD22 in Ramos cells after stimulation with anti-Ig. Lysates were prepared from unstimulated (lane 1) and anti-Ig–stimulated (lane 2) Ramos cells and immunoprecipitated with the 4KB128 antibody to CD22 (Dakopatts, Glostrup, Denmark) (*33*). Precipitates were analyzed by SDS-PAGE and immunoblotting with antiphosphotyrosine. Phosphotyrosyl proteins were detected by fluorography with an intensifying screen. The arrow indicates the 140-kD band.

ters (*22*). $CD22^+$ tonsillar B cells show increased cytosolic free Ca^{2+} and proliferate in response to anti-Ig stimulation, whereas $CD22^-$, surface Ig^+ cells do not (*23*). This implies either that CD22 affects signal transduction in a specific subset of tonsillar B cells or that CD22 is a marker for a subpopulation of B cells that can respond to cross-linking of membrane Ig. In support of the former possibility, treatment of tonsillar B cells with antibodies to CD22 augments the ability of anti-Ig to increase cytosolic free Ca^{2+} and to stimulate B cell proliferation (*24*). Our finding that a considerable fraction of the CD22 molecules is phosphorylated on tyrosine after membrane Ig cross-linking also suggests that CD22 actively participates in signal transduction.

CD22 can function as an adhesion molecule, mediating the binding of B cells to a variety of hematopoietic cells, including B and T lymphocytes, erythrocytes, and monocytes (*21*, *25*). Evidence suggests that the 180-kD form of the tyrosine phosphatase CD45 is a CD22 ligand on T cells (*25*). Another CD22 ligand is expressed on B cells; although its identity is not known, it is apparently the substrate of an α2-6 sialyltransferase (*25*, *26*). The adhesive properties of a number of proteins expressed on the surface of hematopoietic cells are regulated. (i) The activity of lymphocyte function–associated antigen–1 (LFA-1), an integrin, is stimulated by signaling through the antigen receptor in T cells (*27*). (ii) The ability of the gpIIb-IIIa platelet integrin to bind fibrinogen is increased by platelet activation (*28*). (iii) The ability of CD44 to bind hyaluronic acid is increased by the binding of specific antibodies (*29*). It is possible that tyrosine phosphorylation of CD22 modulates its activity as an adhesion molecule, with subsequent consequences for B cell activation.

REFERENCES AND NOTES

1. J. Hombach, L. Leclercq, A. Radbruch, K. Rajewsky, M. Reth, *EMBO J.* **7**, 3451 (1988); J. Hombach, F. Lottspeich, M. Reth, *Eur. J. Immunol.* **20**, 2795 (1990); J. Hombach, T. Tsubata, L. Leclercq, H. Stappert, M. Reth, *Nature* **343**, 760 (1990); J. Weinands, J. Hombach, A. Radbruch, C. Reisterer, M. Reth, *EMBO J.* **9**, 449 (1990); K. S. Campbell and J. C. Cambier, *ibid.*, p. 441; K. S. Campbell, E. J. Hager, R. J. Friedrich, J. C. Cambier, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3982 (1991); A. R. Venkitaraman, G. T. Williams, P. Dariavach, M. S. Neuberger, *Nature* **352**, 777 (1991); K. Ishihara *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 633 (1992); M. Reth, *Annu. Rev. Immunol.* **10**, 97 (1992).
2. Y. Yamanashi, T. Kakiuchi, J. Mizuguchi, T. Yamamoto, K. Toyoshima, *Science* **251**, 192 (1991); A. L. Burkhardt, M. Brunswick, J. B. Bolen, J. J. Mond, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7410 (1991).
3. M.-A. Campbell and B. M. Sefton, *Mol. Cell. Biol.* **12**, 2315 (1992).
4. J. E. Hutchcroft, M. L. Harrison, R. L. Geahlen, *J. Biol. Chem.* **266**, 14846 (1991).
5. _____, *ibid.* **267**, 8613 (1992); T. Taniguchi *et al.*, *ibid.* **266**, 15790 (1991).
6. M.-A. Campbell and B. M. Sefton, *EMBO J.* **9**, 2125 (1990).
7. M. R. Gold, D. A. Law, A. L. DeFranco, *Nature* **345**, 810 (1990); P. J. L. Lane, F. M. McConnell, G. L. Schieven, E. A. Clark, J. A. Ledbetter, *J. Immunol.* **144**, 3684 (1990); M. Brunswick, L. E. Samelson, J. J. Mond, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1311 (1991); B. M. Sefton and M.-A. Campbell, *Annu. Rev. Cell Biol.* **7**, 257 (1991).
8. J. E. Alés-Martinez *et al.*, *Eur. J. Immunol.* **22**, 845 (1992).
9. K. M. Coggeshall and J. C. Cambier, *J. Immunol.* **133**, 3382 (1984); M. K. Bijsterbosch, C. J. Meade, G. A. Turner, G. G. B. Klaus, *Cell* **41**, 999 (1985).
10. S. Nishibe *et al.*, *Science* **250**, 1253 (1990).
11. R. H. Carter, D. J. Park, S. G. Rhee, D. T. Fearon, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2745 (1991); R. H. Carter, D. A. Tuveson, D. J. Park, S. G. Rhee, D. T. Fearon, *J. Immunol.* **147**, 3663 (1991); W. M. Hempel, R. C. Schatzman, A. L. DeFranco, *ibid.* **148**, 3021 (1992).
12. P. J. L. Lane *et al.*, *J. Immunol.* **146**, 715 (1991).
13. L. B. Justement, K. S. Campbell, N. C. Chien, J. C. Cambier, *Science* **252**, 1839 (1991).
14. X. R. Bustelo and M. Barbacid, *ibid.* **256**, 1196 (1992).
15. A. Casillas, C. Hanekom, K. Williams, R. Katz, A. E. Nel, *J. Biol. Chem.* **266**, 19088 (1991).
16. M. R. Gold, L. Matsuuchi, R. B. Kelly, A. L. DeFranco, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3436 (1991).
17. Ramos cells (2 × 10^7 cells per milliliter) were stimulated with goat antibodies to the human immunoglobulin M μ chain (anti-Ig) (Cappel, Durham, NC) (20 μg/ml) for 2.5 min at 37°C. Cells were lysed in an RIPA buffer [20 mM tris-HCl (pH 8.4 at 4°C), 150 mM NaCl, 200 μM Na_3VO_4, 2 mM EDTA, 50 mM NaF, 1% Nonidet P-40 (Sigma), 1% sodium deoxycholate, and 0.1% SDS] at 4°C, and phosphotyrosine-containing proteins were adsorbed to Py 72 mouse monoclonal antiphosphotyrosine [J. Glenney, L. Zokas, M. P. Kamps, *J. Immunol. Methods* **109**, 277 (1988)] coupled to Sepharose 4B (Sigma). Bound proteins were eluted with 40 mM phenyl phosphate in RIPA buffer [A. H. Ross, D. Baltimore, H. Eisen, *Nature* **294**, 654 (1981)]. Approximately 15 μg of protein was obtained from 1 × 10^8 cells. The proteins were precipitated with acetone and redissolved in phosphate-buffered saline. BALB/c mice were immunized intraperitoneally five times at 3-week intervals with 5 to 10 μg of purified protein, allowed to rest 6 weeks, and then injected intraperitoneally and subcutaneously with a total of 160 μg of protein. Spleens of immunized mice were fused with murine Sp2/0 cells [M. Shulman, C. D. Wilde, G. Köhler, *Nature* **276**, 269 (1978)]. For screening, we used hybridoma tissue culture supernatants to precipitate phosphotyrosyl proteins purified, as described, from Ramos cells incubated with 200 μM Na_3VO_4 and 2 mM H_2O_2 for 15 min at 37°C [D. Heffetz, I. Bushkin, R. Dror, Y. Zick, *J. Biol. Chem.* **265**, 2896 (1990)]. The combination of Na_3VO_4 and H_2O_2 stimulates the phosphorylation of most of the same proteins as anti-Ig but to a tenfold greater extent (*18*). Immunoprecipitates were screened by immunoblotting with antiphosphotyrosine (*30*).
18. R. J. Schulte, M.-A. Campbell, B. M. Sefton, unpublished data.
19. Ramos cells (1.5 × 10^8) were incubated for 6 hours in 50 ml of medium containing 5 mCi $^{32}P_i$ and 25% of the normal concentration of phosphate. The cells were stimulated with anti-Ig as described (Fig. 1), and the 140-kD protein was isolated by immunoprecipitation with MARA 2. The precipitated protein was subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie brilliant blue. Staining was quantified by scanning densitometry, and the quantity of p140 present was estimated by comparison with known amounts of bovine serum albumin subjected to electrophoresis on the same gel. The p140 band was excised, and the radioactivity present was measured by scintillation spectroscopy. The specific activity of phosphate in the labeling medium was calculated from the concentration of radioactivity present, as deter-

mined by scintillation spectroscopy, and the concentration of phosphate in the labeling medium, which was ~0.25 mM. We derived the estimate of 1.5 mol of phosphate per mole of protein by assuming that the specific activity of phosphate in protein was the same as that in the labeling medium. This value probably underestimates the extent of phosphorylation.
20. G. L. Wilson, C. H. Fox, A. S. Fauci, J. H. Kehrl, *J. Exp. Med.* **173**, 137 (1991).
21. I. Stamenkovic and B. Seed, *Nature* **345**, 74 (1990).
22. B. Dorken *et al.*, *J. Immunol.* **136**, 4470 (1986).
23. A. Pezzutto, P. S. Rabinovitch, B. Dorken, G. Moldenhauer, E. A. Clark, *ibid.* **140**, 1791 (1988).
24. A. Pezzutto, B. Dorken, G. Moldenhauer, E. A. Clark, *ibid.* **138**, 98 (1987).
25. I. Stamenkovic, D. Sgroi, A. Aruffo, M. S. Sy, T. Anderson, *Cell* **66**, 1133 (1991).
26. I. Stamenkovic, D. Sgroi, A. Aruffo, *ibid.* **68**, 1003 (1992).
27. M. L. Dustin and T. A. Springer, *Nature* **341**, 619 (1989).
28. G. Di Minno *et al.*, *Blood* **61**, 140 (1983).
29. J. Lesley *et al.*, *J. Exp. Med.* **175**, 257 (1992).
30. M. P. Kamps and B. M. Sefton, *Oncogene* **2**, 305 (1988).
31. ———, *Anal. Biochem.* **176**, 22 (1989).
32. W. H. Fischer, D. Karr, B. Jackson, M. Park, W. Vale, *Methods Neurosci.* **6**, 69 (1991).
33. A. Pezzuto, B. Dörken, G. Moldenhauer, P. S. Rabinovitch, E. A. Clark, in *Leukocyte Typing III*, A. J. McMichael, Ed. (Oxford Univ. Press, Oxford, 1987), pp. 361–364.
34. We thank M. Park for technical assistance. Supported by a fellowship from Schering-Plough (M.-A.C.); by the Clayton Foundation for Research, California Division (W.H.F.); and by grants CA 14195 and CA17289 from the National Cancer Institute of the U.S. Public Health Service.

17 June 1992; accepted 2 September 1992

A Surface Protease and the Invasive Character of Plague

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A 9.5-kilobase plasmid of *Yersinia pestis*, the causative agent of plague, is required for high virulence when mice are inoculated with the bacterium by subcutaneous injection. Inactivation of the plasmid gene *pla*, which encodes a surface protease, increased the median lethal dose of the bacteria for mice by a millionfold. Moreover, cloned *pla* was sufficient to restore segregants lacking the entire *pla*-bearing plasmid to full virulence. Both *pla*$^+$ strains injected subcutaneously and *pla*$^-$ mutants injected intravenously reached high titers in liver and spleen of infected mice, whereas *pla*$^-$ mutants injected subcutaneously failed to do so even though they establish a sustained local infection at the injection site. More inflammatory cells accumulated in lesions caused by the *pla*$^-$ mutants than in lesions produced by the *pla*$^+$ parent. The Pla protease was shown to be a plasminogen activator with unusual kinetic properties. It can also cleave complement C3 at a specific site.

Three members of the genus *Yersinia* are pathogenic for humans. Two of these, *Y. enterocolitica* and *Y. pseudotuberculosis*, are transmitted by ingestion and generally cause a self-limiting mesenteric lymphadenitis or ileitis. In contrast, *Y. pestis* is usually transmitted by the bite of infected fleas and produces the highly invasive, fulminant, and often fatal disease known as plague. Despite these differences in mode of transmission and the character of the diseases they cause, *Y. pestis* and *Y. pseudotuberculosis* are so closely related that *Y. pestis* is classified as a *Y. pseudotuberculosis* subspecies (*1*). Thus, it is of interest to compare these two organisms and to examine features unique to *Y. pestis* that could be responsible for its high virulence.

The most prominent genetic difference between these two species is the presence of two unique plasmids, 110 and 9.5 kb in size, in *Y. pestis*. Although the influence of the larger plasmid on virulence is not known, the smaller plasmid has been shown to contribute to the invasive character of plague. Loss of this plasmid increases the median lethal dose (LD_{50}) of *Y. pestis* for mice by a factor of more than a million, but only when the initial infection occurs at a superficial site (such as in subcutaneous injection) (*2*). If the bacteria are delivered to deeper tissues (by intraperitoneal or intravenous inoculation), little effect on lethal dose is observed. Although it would probably be of no benefit to the orally transmitted yersiniae, the ability to invade deeper tissues after superficial inoculation of the host by flea bite is essential to the flea-rodent-flea life cycle of *Y. pestis* in nature: the bacteria must produce bacteremia to ensure infection of new fleas. Thus, acquisition of the 9.5-kb plasmid was probably an essential step in the evolution of *Y. pestis* from the *Y. pseudotuberculosis* line. The acquisition of this plasmid is also probably responsible for the extreme virulence of *Y. pestis* because death in human plague appears to result from the large numbers of disseminated bacteria rather than from the effects of a specific toxin. The proximal cause of death is most likely reaction of the host to "endotoxin," the lipopolysaccharide component common to the outer membrane of almost all Gram-negative bacteria, including the yersiniae (*3*). Small amounts of this material stimulate host defenses; however, large amounts are lethal, producing high fever, disseminated intravascular coagulation, and irreversible shock.

The 9.5-kb plasmid produces at least three products: a bacteriocin called pesticin, lethal for nonimmune yersiniae; a protein conferring immunity to pesticin; and an outer membrane protease known as Pla (*4*). Pla is responsible for two in vitro phenotypes long associated with *Y. pestis*: weak procoagulant activity and the lysis of fibrin clots, the latter probably produced by activation of plasminogen (*5*). In this report we describe the effect of inactivation of the *pla* and *pst* (pesticin) genes on *Y. pestis* virulence.

All of our experiments were conducted with derivatives of *Y. pestis* strain KIM-10, which was initially isolated from a human plague victim in Iran in the early 1960s (*6*). The 9.5-kb plasmid in this strain has been designated pPCP1 (*4*). We constructed a series of isogenic strains containing pPCP1 mutants or cloned fragments and determined their virulence by measuring LD_{50} values in subcutaneously inoculated mice (Fig. 1). All strains in which the *pla* gene was missing or inactivated (including the strain KIM1008, which carries an internal in-frame *pla* deletion) yielded LD_{50} values close to 10^7. In contrast, LD_{50} values for those strains with an active *pla* gene were all less than 50. Because the latter group includes KIM1006, which contains *pla* but no other gene of pPCP1, we can conclude that *pla* is both necessary and sufficient to explain the contribution of pPCP1 to virulence. Inactivation of *pst* had no effect in these experiments.

We determined the dose capable of infecting 50% of the mice (the ID_{50}) by scoring both mice that died and mice that developed significant antibody titers against the *Y. pestis* capsular protein (F1 antigen) as infected. All of the strains, whether *pla*$^+$ or *pla*$^-$, were infectious at very low doses (ID_{50} < 10) (Fig. 1). Many of the mice infected with *pla*$^-$ mutants had lesions at the injection site that persisted until termination of the experiment after 21 days, another indication of established infection. Histological examination of injection sites at 3 and 4 days after infection (Fig. 2) revealed mac-

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