coli with isopropyl β-D-thiogalactopyranoside and coupled to S-agarose resin. Interaction assays were done with GSTD1 purified as described (*12*) or with lysates of D1 HEK293 cells (*16*) solubilized in 25 mM Hepes (pH 7.4), 50 mM NaCl, 10% glycerol, 1% BSA containing 0.5% Nonidet P-40. Resin-bound S-calcyon (50 μg) or S-β-gal (50 μg) and protein targets (GSTD1, 50 μg; D1 HEK293 lysates, 500 μg) or peptides (200 μg) were nutated for 2 hours at room temperature, washed twice in 10 vol of 25 mM Hepes (pH 7.4) containing 50 mM NaCl, and then resuspended in gel loading buffer.

16. D1 HEK293, a human embryonic kidney 293 (HEK293) cell line expressing hD1 receptors, was established by calcium phosphate transfection of pLXSN plasmid containing hD1 receptor cDNA. Stable transfected cells were maintained in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 10% fetal calf serum, and 250 μg of G418 per milliliter. Cells contained a D1 receptor density of 3.1 pmol per microgram of protein as determined by [^{3}H]SCH23390 radioligand binding.
17. pep 421–435 (SVILDYDTDVSLEKI) and pep2 (NEDQKIGIEIIKRALKI) were synthesized with an ABI model 430A peptide synthesizer using the FastMoc procedures and reagents supplied by Perkin-Elmer, Applied Biosystems division, and resuspended in 100 mM Hepes (pH 7.4) at 1 mg/ml.
18. A 0.9-kb Eco RI–Xho I restriction fragment encoding full-length calcyon was subcloned into pCI and pEGFP-C3 expression plasmids. Twenty-four hours after transfection, about 10^6 pCI or pCI-calcyon-transfected D1 HEK293 cells were solubilized in mild lysis solution containing protease inhibitor cocktail. Protein concentrations were determined and adjusted by dilution so that equal amounts of total protein were incubated with rat anti-D1 monoclonal antibody or normal rat serum (diluted 1:100) for 12 hours at 4°C. Immunoprecipitated proteins were obtained by addition of protein A/G agarose slurry. The resin was washed three times with mild lysis solution, and adsorbed proteins were eluted in SDS-PAGE loading buffer.
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20. Cells (2.5×10^5) were seeded on cover slips and transfected with 0.4 μg of plasmid DNA per well using Effectene. Cells were rinsed with HBS [150 mM NaCl, 10 mM sodium Hepes, 10 mM glucose, 2.5 mM KCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$ (pH 7.4)] 16 to 20 hours after transfection and loaded with 5 μM fura-2 AM [G. Grynkiewicz, M. Poenie, R. Y. Tsien, *J. Biol. Chem.* **260**, 3440 (1985)] in HBS at room temperature. After 20 min, cells were washed three times with HBS. Assays were done at room temperature in 1.5 ml of HBS. Drugs were prepared in HBS and manually applied. For EGTA experiments, Ca^{2+}-free HBS was prepared containing 0.25 mM EGTA. Samples were viewed with an Axiovert 135 microscope (×63 objective). Cells transfected with pEGFP- and pEYFP-containing plasmids were identified at 490 nm and selected as areas for analysis. Images were collected with a charge-coupled device camera connected to a silicon graphics workstation and analyzed with Inovision-Ratiotool 4.3.5. Cells were sequentially illuminated for less than 100 ms, first at 340 nm and then at 380 nm. Fluorescence emission at 510 nm was monitored for each excitation wavelength at 10-s intervals. Average pixel intensities within six to eight selected areas (each area corresponded to a single transfected cell) for both wavelengths at each time point were digitized and stored.
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26. Phosphorylation assays were done in triplicate with purified PKC and S-calcyon or S-β-gal in reaction buffer containing 23 mM tris-HCl (pH 7.4), 0.44 mM $MgCl_2$, 250 mM $CaCl_2$, 1 μM ATP, and 1.5 to 2.0 μCi of [γ-^{32}P]ATP supplemented with 1 μg of diacylglycerol and 6 μg of phosphatidylserine. The reactions were stopped after 5 min at room temperature by addition of SDS-PAGE loading buffer and incubation at 80°C for 5 min. Proteins were resolved by SDS-PAGE on 12% acrylamide gels. Gels were dried before autoradiography.
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Epitopes Involved in Antibody-Mediated Protection from Ebola Virus

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To determine the ability of antibodies to provide protection from Ebola viruses, monoclonal antibodies (mAbs) to the Ebola glycoprotein were generated and evaluated for efficacy. We identified several protective mAbs directed toward five unique epitopes on Ebola glycoprotein. One of the epitopes is conserved among all Ebola viruses that are known to be pathogenic for humans. Some protective mAbs were also effective therapeutically when administered to mice 2 days after exposure to lethal Ebola virus. The identification of protective mAbs has important implications for developing vaccines and therapies for Ebola virus.

Ebola viruses cause acute, lethal hemorrhagic fevers for which no vaccines or treatments currently exist. Knowledge about the immune mechanisms mediating protection is limited. The membrane-anchored glycoprotein (GP) is the only viral protein known to be on the surfaces of virions and infected cells and is presumed to be responsible for receptor binding and fusion of the virus with host cells. As a result, Ebola GP may be an important target of protective antibodies. However, the contribution of antibodies to Ebola GP in disease resistance is unclear. Negligible serum titers of neutralizing antibodies in convalescent patients, together with inconsistent results in achieving protection through experimental transfers of immune sera to animals (*1*, *2*), have led to suggestions that antibodies to Ebola GP cannot confer protection to Ebola virus (*3*).

The role of antibodies to GP in protection is further confounded by the observation that Ebola GP occurs in several forms. Transcriptional editing of the GP mRNA is required for production of the virion-associated GP (*4*, *5*). Proteolytic processing of GP results in two products, GP_1 and membrane-bound GP_2, that covalently associate to form a monomer of the GP spike found on the surfaces of virions (*6*). GP_1 is also released from infected cells in a soluble form (*7*). The unedited GP mRNA encodes a secreted glycoprotein (sGP) that is synthesized in abundance early in infection (*4*, *5*, *8*). sGP and GP_1 are identical in their first 295 NH_2-terminal amino acids, whereas the remaining COOH-terminal 69 amino acids of sGP and 206 amino acids of GP_1 are encoded by different reading frames. It has been suggested that secreted GP_1 or sGP may effectively bind antibodies that might otherwise be protective (*5*, *7*).

This study identified protective GP-specific mAbs (*9–11*) that were classified into five groups on the basis of competitive binding assays (*12–14*). Individual mAbs in these five groups were protective against Ebola challenge when administered prophylactically or thera-

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Table 1. Protective efficacy of Ebola GP mAbs.

Competition group*	mAb designation (isotype†)	Day mAb administered‡	BALB/c S/T§ (100 μg)	BALB/c S/T§ (50 μg)	BALB/c S/T§ (25 μg)	C57BL/6 S/T§ (100 μg)
1	13F6-1-2	−1	10/10	7/10	6/10	9/10
	(IgG2a)	+1	10/10	–	–	9/10
		+2	3/10	–	–	2/10
1	6E3-1-1	−1	0/10	–	–	0/10
	(IgG1)					
2	6D8-1-2	−1	10/10	6/10	3/10	9/10
	(IgG2a)	+1	10/10	–	–	9/10
		+2	6/10	–	–	5/10
3	12B5-1-1	−1	6/10	2/10	0/10	6/10
	(IgG1)	+1	8/10	–	–	6/10
		+2	1/10	–	–	1/10
4	13C6-1-1	−1	10/10	7/10	3/10	9/10
	(IgG2a)	+1	10/10	–	–	10/10
		+2	8/10	–	–	9/10
4	12E12-1-1	−1	0/10	–	–	2/10
	(IgG3)					
5	6D3-1-1	−1	9/10	6/10	2/10	8/10
	(IgG2a)	+1	10/10	–	–	9/10
		+2	9/10	–	–	8/10
5	8C10-1-1	−1	0/10	–	–	1/10
	(IgG2a)					
5	3H8-1-1	−1	0/10	–	–	1/10
	(IgG2a)					
–	Diluent (PBS)	−1	0/10	–	–	0/10
		+1	0/10	–	–	0/10
		+2	0/10	–	–	0/10

*Competition groups were determined as described (*12*). In addition to the mAbs shown, group 2 contained two additional protective mAbs (IgG2a) and group 4 contained three additional protective mAbs (IgG2a) that demonstrated efficacy similar to the protective reference mAbs shown. †Monoclonal antibody isotypes were determined by ELISA (*13*) with anti-IgG1 (Zymed), -IgG2a, -IgG2b, -IgG3 (Cappel), -IgM (Kirkegaard-Perry Laboratories), or -IgA (Sigma) heavy chain–specific antibodies. ‡Groups of five mice per experiment were injected intraperitoneally with either 100, 50, or 25 μg of mAb in PBS 1 day before (−1), or 1 or 2 days after (+1, +2) challenge with 300 times the dose lethal for 50% of adult mice (10 plaque-forming units) of mouse-adapted Ebola Zaire virus (*21*). Mice were checked two times a day for 28 days after infection, which is four times longer than the mean day of death of control animals. §S/T, number of mice that survived challenge/total number challenged.

peutically (Table 1). Three of the epitopes bound by protective mAbs are linear sequences on GP_1, whereas the other two are conformational epitopes shared between GP_1 and sGP (Table 2). Ten out of 14 mAbs identified in these five competition groups protected BALB/c mice from a lethal challenge with mouse-adapted Ebola Zaire virus when 100 μg of purified mAb (*15*) was administered 24 hours before challenge (Table 1). Similar results were observed in a second mouse strain (C57BL/6, Table 1). Protection from Ebola challenge decreased when the mAb dose was lowered to 50 or 25 μg (Table 1). For the most effective mAbs, the amount required for protection was within an acceptable human therapeutic dose of 3 to 5 mg/kg.

Some of the mAbs were effective even when administered up to 2 days after challenge (Table 1), after substantial viral replication had occurred (*16*). None of the mAbs were protective when 100 μg was administered 3 days after challenge, when there are high viral titers (*16*) and possibly irreversible damage of cells and organs.

The ability of the mAbs to inhibit plaque formation by Ebola virus (*17*, *18*), a standard assay of virus neutralization, did not always predict their protective efficacy. None of the protective mAbs inhibited plaque formation in the absence of complement. In the presence of complement, only mAbs in competition groups 2 and 4 neutralized the virus (80% at 6.25 μg/ml). Monoclonal antibody 12B5-1-1 (group 3) did not reduce the number of plaques, but did reduce plaque size, suggesting that it restricted subsequent infection of adjacent cells.

Table 2. Epitopes bound by Ebola GP mAbs.

Competition group	Ebola viruses with epitope*	Ebola GPs with epitope†	Epitope sequence on Ebola GP‡	Amino acids§
1	Z	GP_1	ATQV**EQHHRRTDN**DSTA	401–417
2	Z	GP_1	HNTP**VYKLDISEA**TQVE	389–405
3	Z	GP_1	GKLG**LITNTIAGV**AGLI	477–493
4	Z, IC, S	GP_1, sGP	Unknown	Unknown
5	Z, IC	GP_1, sGP	Unknown	Unknown

*Reactivities of mAbs with Ebola Zaire (Z, isolates from 1976 and 1995), Sudan (S), and Ivory Coast (IC) viruses in ELISA (*13*). †Determined by Western blot reactivity with Ebola Zaire 1995 virions or by immunoprecipitation (Fig. 1). ‡Monoclonal antibodies bound two consecutive peptide sequences immobilized on SPOTS membranes. Each peptide was 13 amino acids long and had a 9–amino acid overlap with the preceding and subsequent peptides (*22*). Sequences in bold indicate the 9–amino acid overlapping consensus sequence found on both peptides bound by the mAbs. Peptides containing the entire amino acid sequence shown also competed the binding of mAbs to Ebola virions in ELISA. §Amino acid numbers are based on the GP sequence from GenBank [accession number U23187 (*5*)]. Abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; G, Gly; H, His; I, Ile; K, Lys; L, Leu; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; and Y, Tyr.

When the mAbs were tested for reactivity with the Ebola viruses that are human pathogens, mAbs in groups 1, 2, and 3 bound to the two Zaire isolates that have caused the most devastating outbreaks, but did not bind to the Ivory Coast or Sudan viruses (Table 2). Monoclonal antibodies in these three groups immunoprecipitated GP, but not sGP, from supernatants of cell cultures infected with either Ebola Zaire virus or Ebola GP replicons (Fig. 1) and reacted only with GP_1 in Western blots (*19*). The sequences bound by these mAbs were identified by means of synthetic peptides immobilized on membranes and were confirmed with soluble peptides in competition enzyme-linked immunosorbent assays (ELISAs) (Table 2). These protective mAbs bound linear epitopes within a region of 106 amino acids in the COOH-terminal portion of GP_1. This region is poorly conserved among Ebola viruses and is not shared with sGP. The epitopes bound by mAbs in groups 1 and 2 are separated by only

three amino acids (Table 2).

In contrast, mAbs in groups 4 and 5 immunoprecipitated both GP and sGP from supernatants of infected cells (Fig. 1) but did not bind GP on Western blots under reducing conditions (*19*). These epitopes are therefore discontinuous or require a specific conformation for binding and are located within the NH_2-terminal 295 amino acids that are identical between sGP and GP_1. Monoclonal antibodies in group 4 effectively blocked the binding of mAbs in group 5 (*12*), but reciprocal competition was observed only at high concentrations of unlabeled group 5 mAbs (*19*). All of the mAbs in groups 4 and 5 bound to the Ebola Zaire and Ivory Coast viruses. Furthermore, mAbs in group 4, but not group 5, also bound to Ebola Sudan (Table 2).

These results suggest that it is possible to elicit by vaccination, or produce for therapeutic use, antibodies protective against all Ebola viruses that are pathogenic for humans. Moreover, the idea that an antibody's reactivity with both sGP and GP would render it ineffectual in protection (*5*, *7*) is not supported.

Nonprotective mAbs were identified that bound competitively with protective mAbs in groups 1, 4, and 5 (Table 1). All of the antibodies that were completely protective were of the immunoglobulin G2a (IgG2a) subclasses, whereas the competing nonprotective mAbs in groups 1 and 4 were of the IgG1 or IgG3 subclass. Furthermore, the group 3 mAb (12B5-1-1), which was only partially protective, was IgG1. Thus, antibody subclass may be an important factor in protection. Murine IgG2a binds complement more effectively than IgG1 or IgG3 and varies in its affinity for different Fc receptors (*20*). The subclass of the antibody may therefore affect the ability of the mAbs to resolve Ebola infections either by lysing infected cells through the classical complement pathway or by binding Fc receptors on cellular effectors of antibody-dependent cell-mediated cytotoxicity.

Alternatively, the affinity of an antibody for its epitope, possibly influenced by posttranslational modifications such as glycosylation, may be an important determinant of protective efficacy. For instance, although group 5 consisted of three IgG2a mAbs, only 6D3-1-1 (Table 1) was protective. This mAb bound to Ebola virus at 10-fold lower concentrations than the two nonprotective mAbs (*13*, *19*). In addition, the protective mAb in group 1 was more effective than the nonprotective mAb in competition assays (*19*), suggesting that protective mAbs may have higher affinities for the epitope than nonprotective mAbs.

Monoclonal antibodies to Ebola GP protect immunocompetent animals from lethal Ebola challenge, demonstrating that antibodies are a feasible option for the design of safe and standardized treatments for Ebola infections. However, antibody specificity and the ability to neutralize the Ebola virus in vitro cannot be used as sole predictors of protective efficacy. Protection may depend on the proper specificity, isotype, and/or affinity of the antibody. These observations may explain conflicting data and interpretations regarding the role of antibodies in protection from Ebola virus. Furthermore, the induction of antibodies should not be overlooked when designing vaccines and therapies for Ebola virus.

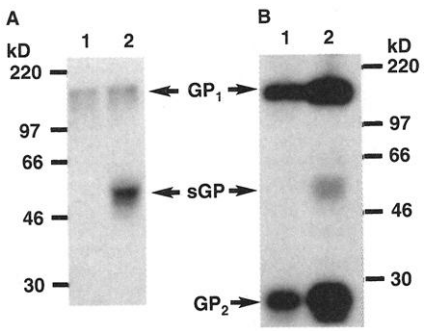

Fig. 1. Immunoprecipitation (*14*) of ^{35}S-labeled Ebola GPs from supernatants of Vero cells infected with (**A**) Ebola GP replicons or (**B**) Ebola Zaire 1995 virus, with either the group 1 mAb 13F6-1-2 (lane 1) or the group 4 mAb 13C6-1-1 (lane 2). Both preparations contained secreted GP_1 and sGP. Disulfide-linked GP_1 and GP_2 constitute the spikes on the virions that are also present in the Ebola-infected preparation (B). The immunoprecipitation of GPs with 13F6-1-2 was identical to that observed with mAbs in groups 1, 2, and 3. Monoclonal antibodies in groups 4 and 5 had reactivities identical to that of mAb 13C6-1-1. GP proteins were resolved under reducing conditions on an 11% SDS-polyacrylamide gel.

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13. ELISAs were performed as described (*14*) with plates coated with sucrose-purified, irradiated virions prepared from the following Ebola strains: Zaire 1995, Zaire 1976 (isolate Mayinga), Sudan (isolate Boniface), and Ivory Coast (#807212, obtained from the Centers for Disease Control). In some experiments, the secondary antibodies were conjugated with alkaline phosphatase, and *p*-nitrophenyl phosphate was used as the substrate. ELISA values were considered positive if they exceeded background values by 0.2 absorbance units.
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22. On the basis of the Ebola Zaire GP sequence (*5*), peptides were synthesized directly on SPOTS membranes by Genosys. Binding of the mAbs to the peptides was performed according to the manufacturer's instructions.

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