Two-state selection of conformation-specific antibodies

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We present a general strategy for identification of conformation-specific antibodies using phage display. Different covalent probes were used to trap caspase-1 into 2 alternative conformations, termed the on-form and the off-form. These conformation-trapped forms of the protease were used as antigens in alternating rounds of selection and antiselection for antibody antigen-binding fragments (Fabs) displayed on phage. After affinity maturation, 2 Fabs were isolated with K_D values ranging from 2 to 5 nM, and each bound to their cognate conformer 20- to 500-fold more tightly than their noncognate conformer. Kinetic analysis of the Fabs indicated that binding was conformation dependent, and that the wild-type caspase-1 sits much closer to the off-form than the on-form. Bivalent IgG forms of the Fabs were used to localize the different states in cells and revealed the activated caspase-1 is concentrated in a central structure in the cytosol, similar to what has been described as the pyroptosome. These studies demonstrate a general strategy for producing conformation-selective antibodies and show their utility for probing the distribution of caspase-1 conformational states in vitro and in cells.

allostery | caspase-1 | phage display | protein conformational change

Protein allostery is a central means to regulate protein function in cells. Allostery is mediated through conformational selection upon binding of different small molecules, biopolymers, or metal ions or through posttranslational modifications. Structural methods give us high-resolution insight into the nature of these conformational transitions in vitro but have limited use for determining the equilibrium distribution of these states in solution or in cells. To expand the tools useful for trapping and analyzing conformational states in enzymes, both in solution and in cells, we developed a general strategy for 2-state selection of conformation-specific antibodies using phage display.

As a test case, we focused on caspase-1, an aspartate-specific thiol protease that is critical for processing of proinflammatory cytokines during the innate immune response (for review, see refs. 1–3). The enzyme is produced as an inactive proenzyme that exists primarily as a monomer in solution (4, 5). Upon innate immune stimuli, the proenzyme is believed to dimerize by binding to scaffolding proteins known collectively as the inflammasome. This triggers proteolytic autoactivation or transactivation, in which the propeptide and an intersubunit linker are cleaved (6, 7). Crystal structures of the mature protease with various small molecules bound show that it can exist in at least 2 conformations (8–10). When an active site inhibitor is bound, the enzyme appears to be in a catalytically competent form (called the on-form) (9). However, binding of covalent disulfide ligand to a central cavity ≈15 Å from the active site stabilizes the protease in an inactive state (called the off-form) (8). This allosterically inhibited state is virtually identical to the apo-form of the enzyme as seen in the crystal structures. The dimeric enzyme shows positive cooperativity [n_{hill} = 1.5 (8)], and mutational studies reveal that only a small set of residues (a "hot-wire") mediates the on-to-off transition between the allosteric and active sites (11). These data support a dynamic activation model for caspase-1 (Fig. 1*A*).

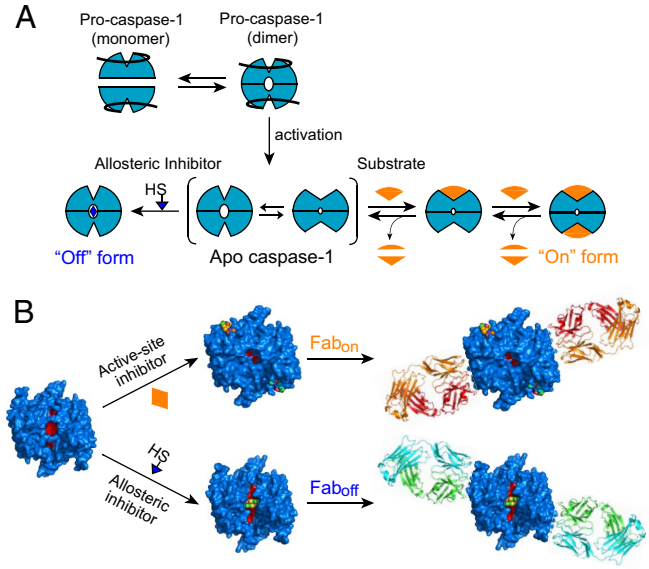

Fig. 1. Model and labeling design. (*A*) Proposed model for the dynamic activation of caspase-1. It has been suggested that in cells procaspase-1 exists primarily as monomer. Upon binding to scaffolding proteins (NALPs, ASC, etc.), procaspase-1 dimerizes and undergoes proteolytic activation. Mature caspase-1 is in equilibrium between off- and on-conformations. Binding of ligands at the active or allosteric site can shift the equilibrium toward the on- or off-state. (*B*) Covalent labeling of apo-caspase-1. Irreversible active-site inhibitors or allosteric compounds were used to trap caspase-1 into a stable conformation for antibody selection.

We wished to generate monoclonal antibodies to each of the on- and off-states to better understand the equilibrium distribution of these states in solution and in cells and to provide probes to localize these forms in cells. We trapped homogeneous forms of the on- and off-states of caspase-1 using the active site inhibitor (Ac-YVAD-cmk) to lock the on-state and compound 34 (1-methyl-3-trifluoromethyl-1H-thieno[2,3-c]pyrazole) to lock the off-state. These conformation-locked forms of caspase-1 were then used as antigens in sorting codon-restricted phage display libraries (12) to generate high-affinity antibody

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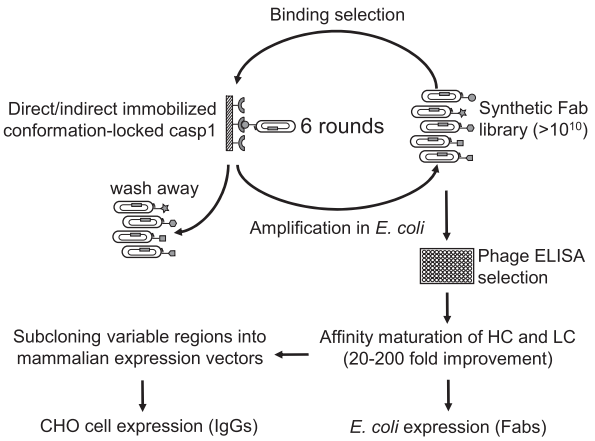

Fig. 2. Selection for conformation-selective antibodies. Six rounds of phage selection were performed for each target. Colonies were picked and tested by spot phage ELISA. Positive clones that showed both good affinity and selectivity were analyzed by DNA sequencing. HC and LC optimization were carried out for affinity maturation. Fabs and IgGs were expressed and purified in *E. coli* and mammalian cells, respectively. See *Materials and Methods* for details.

A

	Fab_{on}			Fab_{off}		
	"on" form	"off" form	apo	"on" form	"off" form	apo
K_D(10^{-9}M)	**2.5**	**>1000**	**330**	**99**	**4.7**	**17**
k_{on}($10^4$$M^{-1}$$s^{-1}$)	66	N.D.	0.8	1.6	135	55
k_{off}($10^{-3}$$s^{-1}$)	1.7	N.D.	2.6	1.6	6.4	9.5

B

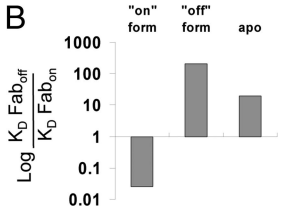

Fig. 3. Fabs are conformation-selective. (*A*) SPR analysis of Fabs by BIAcore. Kinetic analysis was performed by immobilizing different forms of caspase-1 on the CM5 chip and injecting serial dilutions of Fabs. BIAevaluation software was used to fit the recorded sensograms and determine the kinetic parameters. (*B*) The relative difference in K_D of Fabs binding to different forms of caspase-1 was calculated from data in *A*.

fragments (Fabs) that were selective for either the on- or the off-state. The combination of small molecules to trap different molecular conformations coupled with in vitro selection provides a general approach to produce multiple conformation-selective antibodies to biomolecules.

Results

Selection of Conformation-Specific Fabs Using Phage Display. A major challenge to produce conformation-selective antibodies against caspase-1 is to have relatively homogenous preparations of each conformer. To lock caspase-1 in the off-state, we labeled the enzyme with a thiol-containing compound 34 that covalently bound to Cys-331 on the small subunit within the allosteric pocket (8). Similarly, an irreversible active-site inhibitor Ac-YVAD-cmk (CalBiochem) was used to trap the on-state (Fig. 1*B*). The stoichiometric labeling by the different covalent inhibitors to thiols in either the active site or allosteric site was confirmed by LC-MS (Fig. S1).

We used a phage-display strategy that allowed us to maintain the state of antigen throughout the selection process in vitro (Fig. 2). Two synthetic libraries were built on a single antibody fragment framework by introducing limited diversity into the 3 heavy chain CDRs (Complementarity-determining regions: CDR-H1, -H2, and -H3) and the third CDR of the light chain (CDR-L3) (13, 14). A Tyr/Ser focused library was used that explicitly placed Tyr and Ser at solvent accessible positions responsible for antigen recognition (14). A focused Tyr/Ser/Gly/Arg library added further chemical diversity by incorporating Gly and Arg to the binary Tyr/Ser background in CDR-H3 (25% Tyr, 25% Ser, 25% Gly, 25% Arg) (13). The combined naïve library pools contained $>10^{10}$ unique clones.

A total of 6 rounds of selection were performed for both target antigens. After the first 2 rounds, antiselection was enforced by preincubating the amplified phage pool with the off-target conformer. Ninety-six colonies were tested by spot phage ELISA after round 6, and clones showing <20-fold selectivity (based on OD_{450} for the target conformer/OD_{450} for the off-target conformer) were discarded. To exclude Fabs that bound to the active-site or allosteric inhibitors, a second ELISA screen was performed by using a different set of inhibitor-trapped caspase-1 (z-WEHD-fmk for trapping the on-form and compound 11 for trapping the off-form). Only Fabs that showed high selectivity and that bound independently of the specific inhibitors were sequenced. Affinities of Fabs on selected phage were measured by solution-competition phage ELISA (15, 16) (Table S1).

Two different affinity maturation strategies were undertaken to further improve the Fabs (Fig. 2). We focused first on optimizing Fabs for the on-form by partial randomization of all 3 CDR loops on the heavy chain (17). Four clones with affinities ranging from 50 to 110 nM were used as independent starting templates (Table S1). We found that a single amino acid change (M to T) at position 100c in the CDR-H3 resulted in the biggest improvement in affinity (>20-fold). The tightest binder (called Fab_{on}) was chosen for expression and subsequent analysis (Table 1). The same strategy failed to improve the affinity of Fabs for the off-form. Therefore, we shifted our attention to the light-chain CDR loops by randomizing these sequences based on the

Table 1. Sequences of Fab clones after affinity maturation

	CDR-H1										CDR-H2									CDR-H3					
	28	29	30	31	32	33	50	51	52	a	53	54	55	56	57	58	95	96	97	98	99	100	a	b	c
Fab_{on}	N	F	S	Y	Y	S	S	I	S	S	Y	S	S	S	T	S	G	Y	Y	Y	I			G	T
Fab_{off}	S	I	Y	S	S	S	S	I	S	P	Y	Y	G	Y	T	S	Y	S	S	Y	S	Y	Y	A	F
	CDR-L1										CDR-L2									CDR-L3					
	28	29	30	31	32	33	50	51	52	53	54	55					91	92	93	94	95	96			
Fab_{on}	S	V	S	S	A	V	S	A	S	S	L	Y					S	Y	S	Y	P	S			
Fab_{off}	V	V	V	R	Y	L	L	A	S	N	L	A					S	S	A	F	P	L			

Only CDR sequences at positions that were randomized in the libraries are shown. The numbering is according to the nomenclature of Kabat *et al.* (34).

A

	$k_{D(apo\ casp1)}$(nM)	IC_{50}(nM)	EC_{50}(nM)	Ratio(K_D/EC_{50} or IC_{50})
Fab_{on}	330	-	11.7	28
Fab_{off}	17	920	-	1/54

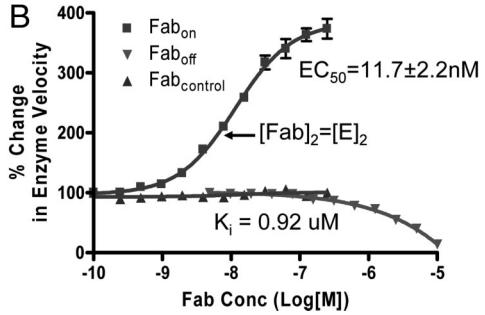

Fig. 4. Activation or inhibition of caspase-1 activity by Fabs. (*A*) Caspase-1 (5 nM) was preincubated with serial dilution of Fabs before assaying with fluorescent substrate Ac-WEHD-fmk at 100 μM concentration. The relative change in enzyme velocity was calculated by dividing the enzyme velocity at any given Fab concentration with the average velocity of enzyme in the absence of Fab. Nonlinear regression was used to fit the curve and calculate EC_{50} and K_i. (*B*) The ratios of $K_{D(apo\ caspase\text{-}1)}$ for each Fab to the corresponding EC_{50} or IC_{50} were calculated from data in *A* and Fig. 3*A*.

A

	IgG_{on}		IgG_{off}	
	pro-casp1	apo casp1	pro-casp1	apo casp1
K_D(10^{-9}M)	**N.D.**	**220**	**130**	**0.38**
k_{on}($10^4M^{-1}s^{-1}$)	N.D.	3.1	35	1460
k_{off}($10^{-3}s^{-1}$)	N.D.	6.9	46	5.5

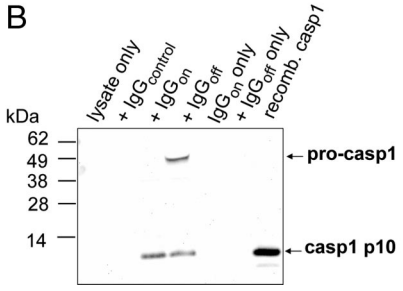

Fig. 5. IgG_{off} binds to procaspase-1. (*A*) SPR analysis of IgGs by BIAcore. Kinetic analysis was performed by immobilizing procaspase-1 or apo caspase-1 on the CM5 chip and injecting serial dilutions of IgGs. BIAevaluation software was used to fit the recorded sensograms and determine the kinetic parameters. (*B*) Immunoprecipitation of procaspase-1 and apo caspase-1 from THP-1 lysates by IgGs. Monoclonal anti-caspase-1-p10 antibody (Calbiochem) that recognizes both procaspase-1 and caspase-1 p10 subunit was used in Western blot analysis.

natural diversity of human kappa light chain sequences in the Kabat database (18, 19). This resulted in >100-fold improvement of affinity for the best clone (called Fab_{off}, Table 1).

Biochemical Characterization of Conformation-Specific Antibodies. We expressed Fab_{on} and Fab_{off} in *Escherichia coli* and purified them by protein-A affinity chromatography. To characterize the binding affinity and selectivity of the Fabs, we tested their interaction against various caspase-1 conformers by surface plasmon resonance (SPR). As shown in Fig. 3*A*, both Fabs bind to their target conformers tightly with low-nM K_D values that were similar to those estimated from solution-competition phage ELISA (12.9 ± 1.1 nM for Fab_{on}-phage; 7.9 ± 0.5 nM for Fab_{off}-phage). Fab_{on} showed no binding to the off-state caspase-1 even at highest test concentration (1 μM) on BIAcore, whereas Fab_{off} bound to the on-state caspase-1 at least 20-fold weaker than to its cognate form (99 vs. 4.7 nM, respectively). The high selectivity demonstrated by the Fabs was mostly contributed by the difference in k_{on} not k_{off}. This suggests these Fabs are selective for the specific conformation of caspase-1.

Given the sensitivity of the Fabs for different caspase-1 conformers, we were intrigued to investigate the conformational state of ligand-free caspase-1. Kinetic analysis using BIAcore showed that Fab_{off} bound to apo caspase-1 with nearly 20-fold higher affinity than Fab_{on} (17 vs. 330 nM, respectively; Fig. 3*A*). Again, most of the difference was in k_{on}, not in k_{off}. Comparing how well ligand-free caspase-1 bound to both Fabs with that of the locked-on and -off forms (Fig. 3*B*), we concluded that caspase-1 is in an equilibrium between the on- and off-states but that the dominant species is much closer to the off-state conformation.

Activation or Inhibition of Caspase-1 with Conformation-Specific Antibodies. We explored the possibility of affecting caspase-1 activity by stabilizing each of the 2 conformational states upon binding to the 2 different Fabs. As seen in Fig. 4*A*, Fab_{on} increased the catalytic activity of caspase-1 by over 3-fold in a dose-dependent manner. The EC_{50} value (11.7 ± 2.2 nM) was close to its K_D for the fully locked on-state conformer (2.5 nM, Fig. 3*A*). In contrast, Fab_{off} functioned as an inhibitor, which prevented substrate binding by trapping the free enzyme in the off-state. The EC_{50} and IC_{50} for activation and inhibition were 28-fold lower or 54-fold higher than their respective K_Ds for binding of the apo-caspase-1 to Fab_{on} or Fab_{off} (Fig. 4*B*). This is consistent with previous studies showing that the enzyme exhibits positive cooperativity (8, 11), where substrate drives the enzyme from the off- to the on-form (Fig. 1*A*).

Effects on Binding to Procaspase-1 in Vitro and in Cells. Previous biochemical studies have suggested that procaspase-1 exists as a monomer (4, 5). To determine the binding of the conformation-specific antibodies to procaspase-1, we expressed and purified a recombinant form of procaspase-1 in which the CARD domain was deleted, called p32 (residues 120–404). SPR analysis showed that neither monomeric Fab was able to bind to p32 (Fig. S2). We converted Fabs into full-length human dimeric IgGs and measured their binding to p32 and to ligand-free caspase-1. Both IgGs bound to ligand-free caspase-1 like their Fab counterparts; however, the binding affinity of IgG_{off} was at least 500-fold stronger than IgG_{on} (Fig. 5*A*). The dimeric IgG_{off} was able to bind to p32 with an affinity of 130 nM, possibly by driving the dimerization of p32. Even though we cannot exclude the possibility that the binding epitope of IgGs might include the cleavage site, which is present in the mature form but not in the pro form, it is less likely because both antigens (on- and off-form caspase-1) we used in the selection had the same cleavage site present. That IgG_{on} could not bind but IgG_{off} suggests p32 exists in a state much closer to the off-state.

To test whether IgG_{off} recognizes full-length procaspase-1 in cells, we carried out immunoprecipitation experiments in cell extracts from human THP-1 cells that have high levels of endogenous procaspase-1. As depicted in Fig. 5*B*, both IgG_{on} and IgG_{off} pulled down caspase-1, whereas only the latter was able to immunoprecipitate full-length procaspase-1. These results align with the binding data above and further suggest that the IgGs are useful tools for studying the specific conformations of caspase-1 in cells.

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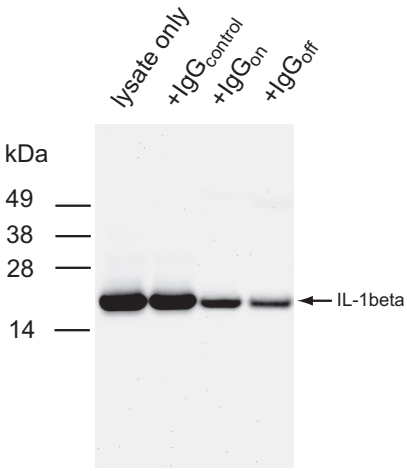

Fig. 6. Inhibition of caspase-1 by IgG_{off} in THP-1 extracts. LPS-stimulated THP-1 cell extracts were incubated with IgGs at 4 °C overnight and probed by Western blot analysis using an antibody specific for mature IL-1ß (Cell Signaling).

To investigate whether the antibodies have similar effects on caspase-1-mediated cleavage in cells, we examined the processing of pro-IL-1ß, a well characterized natural caspase-1 substrate, in the presence of each of the 2 IgGs. THP-1 cells were stimulated by LPS for 4 h to increase pro-Il-1ß expression, and cell extract was prepared as described (7). Western blots probed for mature Il-1ß showed that IgG_{off} significantly blocked caspase-1-mediated cleavage of pro-Il-1ß (Fig. 6). IgG_{on} also inhibited processing of pro-IL-1 ß but to a lesser extent possibly by sterically blocking the larger protein substrate binding. These results suggest that IgG_{off} can effectively inhibit caspase-1 activity in cells by sequestering the enzyme in the off-state.

Localization of Active Caspase-1 Conformer in Cells. The ability of IgG_{on} to bind selectively to the on-form of caspase-1 and not to procaspase-1 makes it potentially useful for tracking caspase-1 activation in cells. THP-1 cells can be differentiated into macrophages with PMA treatment, which then become adherent to glass coverslips. After fixation and mild detergent permealization, IgG_{on} was added as the probe for the on-state of caspase-1. Immunofluorescent microscopy revealed that most of cells stimulated with LPS/ATP showed a general increase in diffuse fluorescent signal (Fig. S3). Intriguingly, ≈10% of these cells contained a single bright, near-spherical structure located in the cytosol (Fig. 7). That we observed this structure only when staining with IgG_{on} and not with IgG_{off} suggests it is highly enriched for the on-state of caspase-1.

Discussion

We present a general strategy for directly obtaining conformation-selective antibodies that was applied to study distributions of on- and off-conformers of caspase-1. The differences in conformation for the on- and off-states as viewed by X-ray crystallography are relatively modest. Thus, that high-affinity antibodies specific for the 2 states could be isolated suggests the wide applicability of this strategy.

The key elements of this approach are the availability of ligands to trap specific conformations of the protein of interest coupled with an in vitro selection method to isolate specific binding proteins. Although we used covalent ligands to lock specific conformations, one could also employ noncovalent ligands, so long as one worked at a high enough concentration to maintain saturation. It should also be possible to trap these alternate target conformations with natural protein-binding partners or using engineered disulfide or chemical cross-linkers.

State-selective binding partners have been identified before. It has been reported that using phage display to isolate peptides specific for estradiol- or tamoxifen-activated estrogen receptor (20). Fabs have also been selected for GTP-bound Ras (21). However, neither study selected for more than 1 state. Recent elegant work by Grutter and coworkers (22) has shown that it is possible to isolate conformation-specific binding proteins to caspase-2 without using conformation-trapped antigens. However, this approach required the assaying of >100 individual clones following selections from a combinatorial library of designed ankyrin repeat proteins (DARPin) to identify the allosteric inhibitors.

In vitro selection allowed antiselection in solution (23) with the noncognate form of the protein, which enabled the dramatic enrichment of selective binders. Although we found phage display to be particularly effective in vitro selection, there are a variety of other methods one could apply, including ribosome display, yeast display, and others (for review, see refs. 24 and 25). Fab display is particularly effective, because it is well established for naïve selections and has been made even more effective recently by using codon-restricted CDR libraries enriched for Ser and Tyr residues (12, 14). However, there is a variety of other scaffolds that could be potentially used, such as DARPins (26), Affibodies (27), fibronectin type III domains (28), zinc finger DNA-binding domains (29), and many others. Moreover, one could imagine using this approach to identify conformation-selective antibodies to other biopolymers such as alternate forms of DNA or RNAs such as riboswitches, etc.

Our data suggest that the Fabs we identified are specific for the conformers of caspase-1 they were selected for. First, at the selection stage, we found strong enrichments for the specific antigen used in the selection, and binders survived the antiselection steps with noncognate antigens. The consensus se-

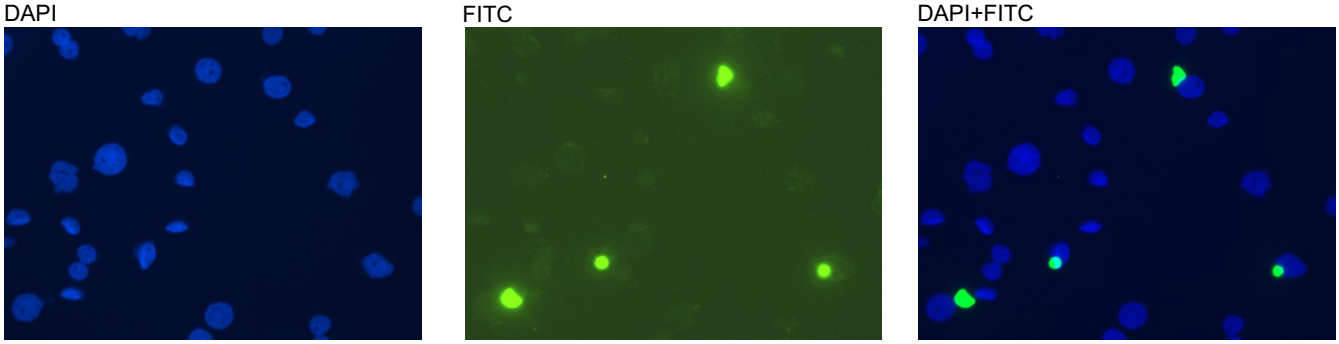

Fig. 7. Probing active conformers of caspase-1 in THP-1 cells. LPS-stimulated THP-1 cells were stained with DAPI (*A*) and IgG_{on} (*B*). The merged image is shown in *C*. Note the spherical body found in ≈10% of cells.

quences for the Fab_{on} and Fab_{off} were very different from each other, and they bound to their cognate antigen with 20- to 500-fold higher affinity than to the noncognate forms. Strong evidence for conformational specificity was provided by the SPR data, which showed that the increased affinity for the cognate antigen was due to a faster on-rate rather than a slower off-rate. Interestingly, the fact that the antibodies can bind to the noncognate form at all suggests each of the inhibitor-locked antigens is dynamic, albeit the inhibitor-locked cognate conformer is much more populated than the noncognate conformer. In addition, both Fabs did not cross-react with caspase-4 and -5, which have the highest sequence similarity to caspase-1 (Fig. S4). We do not know the exact epitopes where these Fabs bind on caspase-1. However, it is very unlikely their epitopes include the inhibitor itself. In fact, the structure of the inhibitor was alternated during positive selection to avoid antibodies that interacted directly with the inhibitor.

The binding kinetics of these Fabs to the apo form of caspase-1 suggests strongly that the enzyme in solution is dynamically interchanging between conformations because the affinity for Fabs is intermediate between the locked forms of caspase-1 (Fig. 3*B*). However, the apo enzyme sits overwhelmingly in the off-form. For example, the enzyme binds with an affinity that is much closer to the locked off-form of caspase-1 than to the on-form. The ratios of K_D values for the Fab_{off} to Fab_{on} vary >4 logs; the unlabeled caspase-1 is within 1 log unit of the locked-off form yet 3 log units away from the locked-on form of caspase-1. Thus, the average ensemble of conformations for apo caspase-1 is intermediate between the on- and off-states but populates the off- over the on-state by a factor of ≈1,000. It is noteworthy that X-ray structures show the conformation of the apo-form of caspase-1 (9) is virtually identical to the off-form generated by the allosteric tethered ligand (8).

The Fab fragments have marked functional effects on the apo caspase-1 that are consistent with previous reports that the enzyme exhibits positive cooperativity (8, 11). The titration of apo-caspase-1 with Fab_{on} causes >3-fold activation of activity (Fig. 4*A*) probably by binding and stabilizing the active conformer. Moreover, the EC_{50} for activation by Fab_{on} (11 nM) is significantly lower than the K_D for binding (300 nM; Fig. 4*B*). These data suggest that the presence of substrate can stabilize the active conformer and thus improve apparent affinity. Likewise, Fab_{off} inhibits the enzyme. The IC_{50} for inhibition (920 nM) is significantly above the K_D for binding of Fab_{off} (5 nM) and reflects the fact that substrate stabilizes the on-form and thus competes for binding of Fab_{off}. We previously showed that labeling caspase-1 with Ac-YVAD-cmk and the allosteric inhibitor are mutually exclusive: binding of the active site inhibitor promotes a conformation that is incompatible with binding of the allosteric inhibitor and vice versa (8). Similarly the Fabs appear to bind to mutually exclusive conformations and away from these inhibitor binding sites. Thus, the enzyme can be allosterically activated or inhibited through binding of conformation-specific antibodies on different surfaces.

One of the advantages of using Fabs is that they are readily converted into IgGs that allow one to study possible avidity effects. Interestingly, the IgG_{off} bound 40 times tighter than Fab_{off} to apo-caspase-1, whereas IgG_{on} bound with nearly identical affinity to Fab_{on}. This may reflect the different position of the binding epitope on caspase-1 and resultant stoichiometry such that 1 IgG_{off} molecule can bind to both subunits of caspase-1 simultaneously, whereas 1 IgG_{on} molecule cannot. The enhancement in affinity seen for IgG_{off} for Fab_{off} is almost entirely due to enhanced on-rate, which may reflect that the dimer has a greater probability of productive binding per collision.

Previous studies indicate that procaspase-1 is monomeric (4, 30), yet very little is known about the conformation of this form in vitro or in cells. We find the IgG_{off} binds modestly to procaspase-1 but neither IgG_{on} nor Fab_{on} nor Fab_{off} bind detectably (Fig. 5*A*). This suggests that the pro-form is much closer to the off-form of caspase-1 in conformation and that dimerizing procaspase-1 by binding to IgG_{off} promotes a conformation more like that of apo caspase-1. Nonetheless, the pro-form has a conformation that is not identical to the off-form because IgG_{off} bound to procaspase with a K_D of 130 nM compared with binding to caspase-1 with a K_D of 0.38 nM. These results were mirrored in the immunoprecipitation experiments (Fig. 5*B*). The IgG_{off} can pull down both procaspase-1 and caspase-1 in THP-1 cells, whereas IgG_{on} can pull down only the mature form.

The additional advantage for converting the Fabs to IgGs was that these can be used with standard immunostaining reagents to localize specific conformations of caspase-1 in cells. In THP-1 cells probed with IgG_{off}, we saw only diffuse staining of the cells whether stimulated with LPS or not (data not shown). In contrast, when THP-1 cells were treated with LPS and stained with IgG_{on}, we found intense staining of a single supermolecular cytosolic structure (1~2 μm in diameter) in ≈10% of the cells. Alnemri and coworkers have reported the existence of a virtually identical supermolecular structure, which they termed the pyroptosome, in 15–30% of THP-1 cells stimulated with LPS using GFP-tagged ASC (apoptosis-associated speck-like protein containing a CARD domain) (30). That we can stain the supermolecular structure only with IgG_{on} not IgG_{off} suggests strongly that virtually all of the caspase-1 is processed to the mature form and rests in the active conformation in the pyroptosome-like structure. This would indicate that caspase-1 is either actively catalyzing proteolysis, or perhaps more likely that binding to the ASC scaffold stabilizes the on-form of caspase-1. In this regard, ASC may function like the on-state antibody; however, its binding site does not overlap with that of IgG_{on}.

Overall, these studies demonstrate a general approach for selecting binding proteins to specific protein conformations using small molecule ligands to lock conformations of interest and phage display to identify conformation-selective antibodies. The Fabs and IgG derivatives were useful for defining and localizing the specific forms of caspase-1 in vitro and in cells. Moreover, this approach could be useful for generating specific inhibitors or even activators of proteins in cells extracts and possibly in cells by using appropriate intracellular antibody delivery technology (31, 32).

Materials and Methods

Caspase-1 Expression and Purification. The p20 subunit (residues 120–279) and p10 subunit (residues 317–404) of wild-type human caspase-1 were separately expressed in *E. coli* as inclusion bodies from a pRSET expression vector (Invitrogen). The purification and refolding of protein from inclusion bodies was performed as described (8). The Cys285Ala mutant of caspase-1 was made by refolding Cys285Ala mutated p20 with wild-type p10 inclusion bodies. A form of procaspase-1 lacking the CARD domain (CARDless procaspase, residues 120–404) was cloned into a pET23b expression vector (Novagen) with a C-terminal His_6 tag and transformed into *E. coli* BL21(DE3) strain. The expression was induced with 0.2 mM IPTG induction for 20 min at OD_{600} ≈0.6. Cell pellets were lysed by 5 passes through a microfluidizer in ice-cold lysis buffer (100 mM Tris, pH 8.0, 100 mM NaCl). The lysate was cleared by centrifugation at 48,500 × *g* for 15 min at 4 °C. The supernatant was first loaded on a 5-mL HisTrap HP column (GE Healthcare), and bound protein was eluted with a 0- to 200-mM imidazole gradient after washing. The eluate were diluted into 20 mM Tris, pH 8.0, 5% glycerol, and loaded on a 5-mL HiTrap Q HP column. The p32 was eluted with a 0- to 0.5-M NaCl gradient and aliquots were frozen immediately in an ethanol-dry ice bath.

Caspase-1 Labeling. To prepare the on-form caspase-1, wild-type caspase-1 was incubated with 4-fold excess of active-site inhibitor (Ac-YVAD-cmk or z-WEHD-fmk) at 4°C overnight in the labeling buffer (50 mM Hepes, pH 8.0, 200 mM NaCl, 50 mM KCl, 200 μM ß-ME). Protein precipitate was removed by centrifugation, and the labeling was confirmed by the mass shift observed by

LC-MS (Waters). To prepare the off-form of caspase-1, a catalytic-inactive caspase-1 Cys285Ala was incubated with 150 μM of the allosteric inhibitor [compound 34 or compound 11 (8)] at 4 °C overnight in the same labeling buffer containing 1 mM ß-ME. For random biotinylation, the off-form of caspase-1 was incubated with 15-fold excess sulfo-NHS-LC-biotin (Pierce) for 45 min at ambient temperature, and the reaction was stopped by buffer exchange using a NAP-25 column (GE Healthcare).

Library Construction and Sorting. We modified the Fab-template phagemid (pV-0116c) (12) to have TAA stop codons in all 3 heavy chain CDRs and the light chain CDR-L3 to reduce wild-type Fab background. For the construction of naïve libraries, the resulting phagemid was used as the ''stop template'' in a mutagenesis reaction with oligonucleotides designed to repair simultaneously the stop codons and introduce designed mutations at the desired sites, as described (16).

In sorting for on-form specific Fabs, the phage pool was cycled through rounds of binding selection with the active conformer of caspase-1 that was directly immobilized on 96-well Maxisorp plate (Thermo Fisher). Bound phage were eluted with 100 mM HCl and neutralized with 1 M Tris, pH 8.0. Phage were amplified in *E. coli* XL1-blue (Stratagene) with the addition of M13-KO7 helper phage (New England Biolabs). In sorting for the off-form specific Fabs, a solution-phase binding strategy was adapted for better control over the selection and anti-selection process. The phage pool was incubated for 2 h at room temperature with biotinylated allosteric conformer before being captured on neutravidin or streptavidin (Pierce) coated Maxisorp plates. The bound phage were then eluted and propagated as described above. After selection, individual clones were picked and grown in a 96-well deep well plate with 2YT broth supplemented with carbenicillin and M13-KO7. The culture supernatants were used in phage ELISAs to identify binding clones (33).

Antibody Purification and Kinetic Analysis by SPR. The phage display phagemid was converted into the Fab expression vector by deleting the sequence encoding for the cP3 minor phage coat protein and inserting a λ terminator sequence (GCTCGGTTGCCGCCGGGCGTTTTTTAT) downstream of the stop codon at the end of C_{H1} domain. Fab protein was secreted from *E. coli* 34B8 strain transformed with individual plasmids in low-phosphate medium at 30 °C for 26 h, as described (18). To generate IgG proteins, the variable domains were subcloned into vectors designed for transient IgG expression in CHO cells (18). Fab proteins were purified with protein A affinity chromatography and IgG proteins were purified with protein G affinity chromatography.

Kinetic binding analyses were performed by surface plasmon resonance (SPR) using a BIAcore 3000 (GE Healthcare). Ligand-bound or free caspase-1 dimers were immobilized on CM5 chips and serial dilutions of Fabs or IgGs were injected. Binding responses on flow cells with immobilized caspase-1 variants were corrected by subtraction of responses on a blank reference flow cell. A 1:1 Languir model in BIAevaluation software (GE Healthcare) was used for fitting the sensograms and the K_D values were calculated from the ratios of k_{off}/k_{on}.

Immunoprecipitation from THP-1 Cell Extracts. THP-1 cells were grown to a density of 1×10^6 cell/ml and harvested by centrifugation at $150 \times g$ for 5 min. Cells were lysed by Dounce homogenizer in ice-cold buffer (20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, 0.1 mM PMSF, and the Roche protease inhibitor mixture). Cell lysates were cleared by centrifugation at $500 \times g$, $3{,}000 \times g$, and $22{,}000 \times g$ for 10 min each. Aliquots were incubated with or without IgGs overnight at 4 °C and immunocomplexes were recovered by protein-G agarose beads. Presence of procaspase-1 and caspase-1 were visualized by Western blot analysis.

Immunofluoresence Microscopy. THP-1 cells were grown to the density of 5×10^5 cell/ml and differentiated with 0.5 μM PMA for 3 h and allowed to attach to no. 1½ glass coverslips overnight. Cells were treated with 1 mM LPS for 2 h followed by 5 mM ATP for 30 min before fixation and mild detergent permeabilization. After blocking with 10% BSA for 1 h, IgG_{on} was added at 100 μM concentration for 1 h. After 3 washes with PBS + 0.1% Triton X-100, the cells were stained for 1 h with Alexa Fluor 488 conjugated goat anti-human IgG antibody (Invitrogen). Cells were washed 3 times and mounted with ProLong Gold containing DAPI (Invitrogen). Images were recorded on a Nikon 6D High-Throughput Microscope equipped with a Photometrics Coolsnap HQ2 Camera.

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