Helical Structure of the Needle of the Type III Secretion System of *Shigella flexneri*

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Running Title: Helical Structure of Shigella TTSS III needle
Summary

Gram-negative bacteria commonly interact with animal and plant hosts using type III secretion systems (TTSSs) for translocation of proteins into eukaryotic cells during infection. Ten of the twenty-five TTSS encoding-genes are homologous to components of the bacterial flagellar basal body which the TTSS needle complex morphologically resembles. This indicates a common ancestry although no TTSS sequence homologues for the genes encoding the flagellum are found. We here present a ~16Å structure of the central component, the needle, of the TTSS. Although the needle subunit is significantly smaller and shares no sequence homology with the flagellar hook and filament, it shares a common helical architecture (~5.6 subunits per turn, 24Å helical pitch). This common architecture implies that there will be further mechanistic analogies in the functioning of these two bacterial systems.
Introduction

Gram negative, enteropathogenic bacteria cause a wide variety of diseases, ranging from relatively harmless infections to life threatening illnesses (1). They account for more than 3 million deaths each year, mostly amongst children and immuno-compromised adults in developing countries (2). Infections usually occur where hygiene is poor as the major route of infection with *Salmonella*, *Escherichia*, *Yersinia*, and *Shigella* spp. is the consumption of contaminated food (3) and water (4). Despite significant differences between the distantly related genera, a common macromolecular system, the type III secretion system (TTSS or secreton), is the basis of each infectious cycle. This system consists of more than twenty proteins that form a macromolecular assembly which delivers the bacterial virulence effectors not only across the bacterial inner and outer membranes but also directly into the host cell. The TTSS is well conserved amongst these bacteria whilst the specific properties of the effectors and hence, the resulting symptomatic effects on the host organism vary widely (5). TTSSs are not constitutively active but are activated to secrete by signals that vary between the different genera but seem to ultimately derive from physical contact between the bacterium and its host cell. Understanding how an activation signal is transmitted in such a complex macromolecular assembly and how it results in secretion is one of the major questions to be answered.
Ten of the twenty-five TTS-encoding genes show strong similarity to those that encode the flagellar basal body, indicating a common ancestry (5). Insights into the morphology of the system to date are derived from electron microscopy that reveals a supramolecular structure grossly resembling the flagellar hook-basal body complex (6-8). The major TTSS structure seen is termed the “needle complex” and spans the inner and outer bacterial membranes with a basal body into which a “needle” (~70Å in diameter, traversed by a central 20-30 Å wide channel) is inserted that protrudes about 500 Å into the extracellular space (8,9). Activation of the *Shigella* TTSSs for secretion seems to require contact of the needle tip with the host cell limiting membrane (7). It is particularly difficult to understand how such physical contact leads to a change at the cytoplasmic face of the basal body, ~ 800Å away, and results in secretion of proteins through the structure. Gross structural homologies can therefore be added to the genetic resemblance to flagella noted above. Common ancestry has been used to gain insights into the functioning of the TTSSs on the basis of the well-studied flagellar system (10). However, TTSS homologues for several of the key flagellum building blocks, notably hook protein and flagellin, are lacking. Their absence combined with a lack of any detailed knowledge of the TTSS structures involved, means that the hypothesis of common mode of biological function remains unproven.

This hypothesis implies that the needle of the *Shigella* TTSS will be made up by a helical arrangement of the 10kD protein MxiH (previously identified to be the major needle component (9,11)) with an architecture similar to that
shared by both the flagellar hook and filament. MxiH, however, shows no sequence homology to either the hook or filament subunits and is, in fact, a much smaller protein being ~ 1/5 the molecular weight of either of the flagellar proteins. Homology between these two systems leads to the hypothesis (10) that signal transduction will occur via changes in the architecture of the needle since it is known that the flagellar filament is able to switch between different helical forms (12,13). To test this predicted homology, we have used X-ray fibre diffraction and electron microscopy based 3D reconstruction techniques to determine the structure of the Shigella flexneri needle at ~16Å. We find that, as the hypothesis predicts, the MxiH subunits making up the needle are arranged in a helical fashion to form an extended cylindrical structure with a central channel. The parameters that describe the geometry of the helix are very similar to those of the flagellar hook (14) and filament (15), providing strong support for the hypothesis that these two systems share a common architecture and thus common functional mechanisms.

**Experimental Procedures**

**Bacterial Strains & Growth**

The *Shigella flexneri* serotype 2a non-polar null mutant for *mxiH* strain harbouring pKT001 which encodes the cloned *mxiH* gene under an IPTG inducible promoter (gift of Prof. Chihiro Sasakawa, Tokyo Univeristy, Japan; (11)), hereafter termed *mxiH/mxiH*+++, is plated on a Congo red culture
plate containing kanamycin (50µg/ml), ampicillin (100µg/ml) and trimethoprim (5µg/ml) as selecting antibiotics. Within a week of plating the strain, a single, Congo red positive colony is selected and grown at 37°C for 8-10h in 60ml Trypticase Soy Broth (TCSB), plus antibiotics as above. Using this culture as inoculate, it is diluted 1/100 into the main culture volume of TCSB (plus antibiotics). After induction with 1mM IPTG at the time of inoculation, the culture is incubated overnight at 37°C to obtain maximum bacterial density.

**Preparation of Purified Needle Samples**

10% weight per volume of PEG6k (BDH Ltd, cat # 443915V) is added to the culture to precipitate free needles in the media and the culture is cooled on ice as soon as the PEG has dissolved. Cells (and precipitated needles) are pelleted at 2000g (all centrifuge runs at 4°C) in swing-out buckets. The supernatant is discarded and the pellet re-suspended in 1/100 th of the initial culture volume of PBS. The cell-suspension transferred to a 40ml Dounce glass-glass tissue grinder (“tight” pestle, manufactured by Wheaton, Millville, N.J.) that shaves needles from the cell surface through the exertion of sheer forces. Examination of the sample in an electron microscope shows that 60 cycles of up and down “grinding” is sufficient to remove the majority of needles from the bacteria while keeping most cells intact.

The total volume of the suspension is adjusted with PBS to 1/50th of the initial culture volume and centrifuged at 2000g. The pellet is discarded and the supernatant spun at 12000g to remove remaining macromolecular contaminants. TCA protein precipitation followed by SDS page analysis and
EM confirm that the supernatant contained MxiH (in the form of long needles) free from major contaminants. Finally, needles are precipitated by adding PEG6k and NaCl to a final concentration of 10% and 100 mM respectively. Following 60 min incubation on ice, needles are pelleted at 27,000g. After discarding the supernatant, the small opaque needle pellet is re-suspended in approximately 0.5ml buffer (roughly 1/10,000th of the initial culture volume) at physiological pH (10mM Tris pH 7.4) when it becomes translucent and stored at 4°C. This protocol allows ~5mg of MxiH (in the form of intact needles) to be purified from a culture of 6l of *Shigella mxiH⁻⁻⁻/mxiH⁺⁺⁺* grown overnight.

**X-ray Fibre Diffraction Methods**

Oriented samples of TTSS needles were prepared by a modification of the method of Yamashita et al (16,17). Briefly, 200µl of needle sample (typically with a concentration ~8mg/ml) is diluted ~2X in a salt free Tris-buffer at pH 7.4 and needles concentrated by centrifugation overnight at 10,000 g in a swing-out rotor containing a long thin tube (Beckman Rotor SW50.1/55 using 5x41mm tube and suitable adapter). The supernatant is then carefully removed and 10µl aliquots of the viscous, translucent, pellet are transferred into quartz capillaries with a 0.7mm internal diameter. The capillaries are centrifuged at 2000g for 8h-24h in a swing-out rotor to concentrate the needles at the bottom of the capillary. Careful removal of the liquid above this
region reduces dilution effects and increases sample stability. Capillaries were allowed to order for 1 – 3 weeks at room temperature within the bore of a 15 T magnet (Magnex Scientific, Yarnton, Oxford). Capillaries were then mounted in the beam at station 14.2 of the SRS, Daresbury and images collected on an ADSC scanner using \( \lambda = 0.96 \) Å, \( d = 407 \) mm, \( \Delta \phi = 0 \) degrees and exposure=300 s.

**Electron Microscopy Data Collection**

**Negative staining of isolated needles for electron microscopy:** Purified needles were diluted 1/250 in 10mM Tris, pH 7.5. An aliquot of 5\( \mu l \) was deposited for 1 minute on glow-discharged carbon-coated copper grids. After removal of the excess liquid with filter paper, the sample was stained with a drop of 2% uranyl acetate (pH 7.5).

**Observation and image acquisition:**
The needles were observed with a defocus value of -700 nm in low-dose electron beam conditions \( (\approx 10 \text{ e}^-/\text{Å}^2) \) on a Philips CM12 TEM at 120 kV. Micrographs were recorded at 60,000x magnification on Kodak S0163 films.

**Digitization and image processing preparation**
Images were scanned on a Eurocore Hi-SCAN rotating drum scanner at 1600 dpi resolution. Individual filaments were selected using EmTool (http://ncmi.bcm.tmc.edu/ncmi/software), and image analysis was carried out using SPIDER/WEB (18) software package. 3D reconstructions were done.
within SPIDER using the Iterated Helical Real Space Reconstruction Method (IHRSR) (19).

**Image Processing and Electron Microscopy Reconstruction**

Approximately 10,000 segments (each 100 pixels or ~ 265 Å long) were extracted from micrographs of the mutant filaments, and roughly 5000 segments from wild-type needle complexes (9). The averaged power spectrum was computed for each group of images after only allowing rotations of the individual segments to make the filament axis vertical. This is because the power spectrum is invariant under translations, surmounting the need to align particles. The intensities of the power spectra from each individual segment were simply added to produce an averaged power spectrum. Resolution was determined by generating two reconstructions, each from half of the image segments, and comparing the correlation of Fourier coefficients from these two reconstructions within resolution shells. The coefficient of correlation dropped below 0.5 at a resolution of 12.5 Å, but a significantly more conservative value of 16 Å was used for filtering the final volume.

**Results**

**Purity of the Needle preparation allows Biophysical Studies**

The prerequisite for the majority of biophysical techniques is a highly purified and concentrated sample of the structure to be studied. To facilitate such studies of the *Shigella flexneri* needles we used a *Shigella mxiH*- strain
harbouring a plasmid carrying mxiH under an inducible promoter (hereafter called mxiH-/mxiH+++). This strain can be induced to hugely over-express the needle subunit MxiH. Over-expression of MxiH produces a phenotypic change in the TTSS with the mean needle length changing from ~500Å in the wild type bacteria to ~3,000Å in the mutant (11). Using the protocol described above needles (3,000Å in length on average) could be shaved from the mutant bacteria and purified to greater than 95% homogeneity as demonstrated by SDS-PAGE and electron microscopy analysis (Figure 1).

X-ray Fibre Diffraction Analysis

X-ray fiber diffraction (XRFD) can be a useful method for determining helical parameters of filamentous assemblies, as well as for obtaining constraints in building pseudo-atomic models of helical polymers when only the atomic structure of a monomer is known (20). Unfortunately, no atomic structure exists yet for MxiH. However, XRFD has been used on the needles to provide information that is complementary to that obtainable by electron microscopy (see below). The key to recording high resolution XRFD data is the preparation of a highly ordered, highly concentrated sample of the macromolecular complex to be studied, i.e. preparation of the biological molecule in the form of a liquid crystal. We used a variation on the method of Yamashita et al (16,17) which uses repeated centrifugation and equilibration of the samples in a high magnetic field to produce highly ordered needle samples (see materials & methods). Exposure of these samples to high intensity X-rays allowed for the collection of patterns such as that shown in
Figure 2. All patterns showed the diffraction expected of a helical assembly, confirming that MxiH assembles in a helical fashion to produce the *Shigella* needle. The parameters needed to completely define the helical symmetry are the axial rise (along the filament axis) per subunit, and the azimuthal rotation (about the filament axis) per subunit. The axial rise per subunit gives rise to a reflection in the XRFD pattern that is on the meridian, and the first meridional reflection was observed at a spacing of ~1/4.3 Å (Figure 2c). Strong near-meridional reflections were also observed at a spacing of ~1/24 Å and 1/5.2Å. The full interpretation of this pattern comes from using electron microscopy.

**Reconstruction of the Needle Structure from Electron Micrographs**

To obtain more detailed structural information about the needle we collected a series of electron micrographs from negatively stained specimens. Attempts to use conventional methods of helical image analysis and three-dimensional reconstruction (21) were not successful, due in part to the very weak contrast provided by these thin polymers. Long segments could not readily be found that consistently showed the multiple layer lines needed for Fourier-Bessel methods. Instead, a single-particle approach was used (19), based on analyses of overlapping segments extracted from the micrographs (where each segment was ~265 Å long for the mutant and ~220 Å long for the wild-type). The averaged power spectrum derived from these segments (Figure
2b) is the computational equivalent of the XRFD pattern, since the diffraction intensities have been added from specimens that have been aligned only to have the filament axes oriented in the same direction. Three layer lines with spacings of ~ 1/(58 Å), 1/(37 Å) and 1/(24 Å) were seen in both the average spectrum and in the XRFD pattern (Figure 2). The 1/(24 Å) layer line had peak intensities that were near-meridional, suggesting that it arose from a 1-start helix. A reference-free average (22) of these segments (data not shown) gave rise to a transform showing these same three layer lines. The 1/(24 Å) layer line was determined to be odd, and must correspond to a Bessel order of ±1. The strongest layer line was at 1/(37 Å). Analysis of this layer line showed that it was also odd, and most likely corresponded to a Bessel order of ±5. Similarly, the layer line at 1/(58 Å) was determined to be even, and most likely corresponded to a Bessel order of ±6. The only indexing scheme possible would involve the layer lines at 1/(58 Å) and at 1/(24 Å) arising from helices with the same hand, with the layer line at 1/(37 Å) arising from a helix with the opposite hand. We are unable to determine the handedness of this structure from these data and have therefore assigned a hand based on earlier reconstructions of the flagellar hook and major filament forms – right-handed 1-start helix, left-handed 5-start helix (14,15).

A crude three-dimensional reconstruction was generated by Fourier-Bessel methods from the reference-free average, and this was used as an initial model for the Iterative Helical Real Space Reconstruction (IHRSR) method (19). This method allows for the precise determination of helical symmetry during the course of many cycles where the symmetry is freely
allowed to change, and in this case converged to a structure with ~5.6 subunits per turn of a 24 Å pitch helix after starting from the preliminary model. A confirmation of this reconstruction was that the transform of the image generated from the projection of the structure was found to closely match both the averaged power spectrum and the XRFD data. Since generating either the averaged power spectrum or the XRFD patterns involved no assumptions about symmetry, this is a strong check on the reconstruction. The use of very different indexing schemes to generate the starting model either led the IHRSR cycles to diverge, with the program terminating when the axial rise per subunit became less than zero (when starting with 7.5 subunits per turn), or led to a three-dimensional reconstruction whose transform did not match the averaged power spectrum or XRFD pattern (when starting with 3.5 subunits per turn). Figure 3 shows the helical symmetry parameters as a function of cycle number during the course of iterations starting from ~5.2 subunits per turn. The parameters converge back to approximately the same value obtained when the procedure is started with ~5.6 subunits per turn. Most importantly, the results of the IHRSR method predict an axial rise per subunit of ~ 4.2 Å, well beyond the resolution of the EM images. This is the actual rise (within the level of error introduced by uncertainties in the magnification of the EM images; ~2%) seen in the XRFD pattern, as judged by the meridional intensity corresponding to this spacing (Figure 2c). This can completely exclude alternate indexing schemes that would have ~3.6 or ~7.6 subunits per turn of the 24 Å pitch helix, predicting an axial rise of 6.7 Å or 3.2 Å, respectively.
Reconstructions were made from long needles (Figure 4 a & b) and also from wild-type needles (segments extracted from micrographs of intact purified “needle complexes” prepared as in (9) – data not shown). The helical parameters defining both reconstructions converged to similar values (Table 1) indicating that the architecture of the needle is essentially unaltered by over-expression of MxiH. We have conservatively estimated the resolution of the reconstruction to be ~16 Å, even though a power spectrum generated from this shows a layer line at 1/14 Å that is in good agreement with the layer line observed at this spacing in the XRFD pattern (Fig. 2a). The needle is revealed to be a cylinder of approximate outer diameter 70 Å traversed by a central canal of ~20 Å diameter. The 3D model shows distinct subunits connected by weaker density in the helix. Assuming that each subunit corresponds to a single copy of MxiH, we can determine an expected molecular volume assuming a partial specific volume of protein of 0.75 cm³g⁻¹. The reconstruction is reasonable when a threshold is chosen that encloses this volume. If a threshold is chosen that encloses a much smaller or larger volume, the reconstruction is no longer reasonable (e.g., connectivity is lost, the stain filled volume in the centre is eliminated, etc.) suggesting that there is a single copy of MxiH per asymmetric unit.

Discussion

Common ancestry of the TTS and flagellar systems led to the proposal (10) that there must be TTSS homologues of either flagellin and / or the hook
proteins. Our data show that the needle has the same architecture (~5.6 subunits per turn, ~24Å helical pitch – Table 1) as the flagellar filament (12,15) and hook (14) implying that MxiH and the flagellar hook protein and flagellin must be structural, although not sequence, homologues of one another. This is somewhat surprising, given the huge difference in the size of these two proteins (MxiH is 83 amino acids long, flagellin is 494 aa and the hook protein 402 aa). However, the 3D reconstruction of the flagellar filament (13,15) shows homologies other than similarities of helical parameters to our needle structure. Briefly the flagellar filament structure can be divided into a series of different domains, D0 – an inner tube of ~70Å diameter, with a central channel of ~20Å, D1 the outer tube which surrounds D0 and has an outer radius of ~130 Å and D2 and D3 which project outwards from the helical axis. Fitting of an X-ray crystallographic structure for flagellin into this EM reconstruction (13) allowed Yamashita et al. to determine that the D0 domain was made up of the N and C termini of flagellin (which were not present in the X-ray model as they needed to be truncated to prevent the protein from polymerising). The size and helical architecture of the inner, D0, tube is very similar to the structure we present here for the *Shigella* needle. The question therefore arises as to whether the *Shigella* protein MxiH shares any homology with the termini of flagellin? Although we cannot detect any sequence homology, there is some degree of homology at the level of predicted secondary structure (using programs such as PredictProtein/PHDsec (23-25) and JPred (26)) since we find that the N and C termini of MxiH and flagellin (as well as those of the periplasmic rod, hook and filament junction
proteins) give strong predictions for $\alpha$-helices at their termini. Our data therefore suggest that the needle represents the minimum core required to build a helix of this size with this architecture. In contrast, the flagellin subunit contains a large inserted domain between the N and C termini that is not required for helix formation but presumably relates to functions strictly required for flagellar filaments.

Extending the homology with the flagellar system it was proposed that the assembly of MxiH into the *Shigella* needle would require the presence of a “cap” at the needle tip (10). Located at the end of the assembling structure the cap would aid construction by forcing consecutive subunits into the correct position within the helix (27). This cap protein will be present in the needle complex at such low molar ratio to MxiH (and is likely to be of a similar size) that it is not surprising that we do not biochemically detect it in our purified needles. Further experiments will be required to address this issue.

The hypothesis of common ancestry is primarily attractive in providing a mechanism by which secretion may be switched on by the tip of the *Shigella* needle contacting a host cell (10). The mechanism by which a signal is propagated from the needle tip to the ATPase, putatively located at the base of the basal body some 800Å distant is difficult to understand on a molecular level. The flagellar filament is able to switch helical forms depending on the direction of motor rotation or in response to the chemical environment (28). Switching is thought to be linked to small changes in the conformation of the flagellin subunit leading to gross architectural changes in the filament as a whole (12). This, in turn, leads to changes in the
supercoiling of the filaments within the flagellum and thus transmits the change in motor direction to the rotating flagellum. The idea that this helical architecture might be poised to switch between different conformational states suggests that the effect of contact of the needle with the host cell may be to switch the helical state of the needle – thus producing a signal which is, almost instantly, transmitted to the basal end of the needle and the proteins associated with the basal body.

Whilst this functional hypothesis remains to be proven, our structure, in demonstrating that the Shigella needle equates to at least the D0 domain of the flagellar filament, provides strong support to the hypothesis that contact-activated secretion by Shigella is sensed by changes in the architecture of the needle itself. This remains to be confirmed by studies of activated needles – however these are likely to be difficult, as preparation of homogenous, highly concentrated samples of activated needles presents many experimental challenges that will need to be overcome.

We can now combine all structural information about the Shigella needle to present the model for the Shigella TTSS shown in Figure 4c. This illustrates the global assembly of the TTSS and reinforces the structural similarities to the flagellar hook-basal body complex raising interesting questions on how substrates are recruited, partially unfolded and secreted. In particular, understanding the nature of the interfaces between the basal body and the needle, and that between the needle and its putative cap, which may allow a signal to be transduced to produce secretion, remains an interesting challenge.
Acknowledgments:

We thank Shin-Ichi Aizawa (Utsunomiya, Japan) for suggesting the needle purification protocol based on his experience in purifying bacterial flagella (and testing it with us). M.E.M. Noble for repeated extensions to his program AESOP to allow construction of these figures. F.S.C. is funded by a Wellcome Trust Studentship, A. B.’s lab is supported by the Guy G. F. Newton Cephalosporin Trust Senior Research Fellowship, a Career Transition Grant from the NIH (K22 AI01847) and the Sasakawa Fund at the Oriental Institute (Oxford). K. K. is the recipient of an Edward P. Abrahams Cephalosporin Trust D. Phil. studentship. This work was also supported in part by NIH GM66771 (to E.H.E.). The authors confirm that they have no conflicts of interest with regards to the data presented here.
References

Figure Legends

**Figure 1** (A) Electron micrograph of a preparation of needles in negative stain. (B) SDS-PAGE analysis of needles shaved from the surface of the *mxiH−/mxiH+++* *Shigella flexneri* mutant. Lane 1 shows a preparation of *Shigella* long needles, lane 2 markers with masses as indicated.

**Figure 2** (A) Half of the central region of X-ray fibre diffraction pattern from aligned *mxiH−/mxiH+++* long needles taken at station 14.2 of the SRS, Daresbury (see Materials and Methods). (B) Half of the averaged power spectrum generated from 5402 partially overlapping segments extracted from images of 108 negatively stained *mxiH−/mxiH+++* long needles. For both images the peaks corresponding to specific layer lines are indicated with the layer line spacings and indices (determined as in text) shown. (C) XRFD pattern as shown in (A), but displaying the high-resolution layer lines discussed in the text.

**Figure 3 Convergence of IHRSR helical parameters.**
Convergence of the helical pitch, axial rise and azimuthal rotation as a function of iteration are shown for one test of images from the *mxiH−/mxiH+++* long needles. In this example, the helical parameters were initially chosen to be significantly different from the final value determined by other such tests.

**Fig 4 Various views of the 3D reconstruction** All images produced using AESOP (M.E.M. Noble, unpublished program). (A) and (B) show a top view and side view (respectively) of the 3D reconstruction of the *Shigella flexneri* needles obtained as described in the text. The electron density is thresholded as described in the text to generate the solid surface (C) is a composite image giving a model for the structure of the needle complex in the bacterial membranes using the needle reconstruction presented here in combination with the earlier reconstruction of the basal body of the needle complex (9).
Table 1

Helical Parameters determined from the *Shigella* needle reconstructions (as described in the text) and comparison with those obtained for the flagellar filament (13) and straightened hook (14).

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<td>Flagellar Hook (14)</td>
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Figure 1
Figure 2
Figure 3 Convergence of IHRSR helical parameters
Figure 4 Various views of the 3D reconstruction

A

B

C

~25Å

~70Å

~500Å
Additions and Corrections


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Frank S. Cordes, Kaoru Komoriya, Eric Larquet, Shixin Yang, Edward H. Egelman, Ariel Blocker, and Susan M. Lea

Page 17107, Acknowledgments: The name of our collaborator was incorrectly listed. The correct name is David DeRosier.


Angiotensin II enhances adenylyl cyclase signaling via Ca2+/calmodulin. Gq-Gs cross-talk regulates collagen production in cardiac fibroblasts.

Rennolds S. Ostrom, Jennifer E. Naugle, Miki Hase, Caroline Gregorian, James S. Swaney, Paul A. Insel, Laurence L. Brunton, and J. Gary Meszaros

Page 24465: In Fig. 5 of this paper, we analyzed the localization of natively expressed isoforms of adenylyl cyclase (AC) in buoyant, caveolin-rich fractions from rat cardiac fibroblasts isolated on a discontinuous sucrose gradient. Fig. 5 shows and under “Results” we state that immunoreactivity to AC8 and AC9 was not detected. However, under “Discussion,” we mistakenly stated that we observed AC8 immunoreactivity in non-caveolin-rich fractions. While a minority of our studies revealed a small amount of AC8 immunoreactivity in non-caveolin-rich fractions, our results were neither definitive nor consistent enough for us to conclude that AC8 is expressed in non-caveolin-rich fractions. Therefore, we conclude that AC8 protein is not detectable in cardiac fibroblasts by the methods that we have employed.

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