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Nucleic acid chaperone activity of retroviral Gag proteins

Alan Rein

HIV Drug Resistance Program; National Cancer Institute-Frederick; Frederick, MD USA

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Abbreviations: MA, matrix; CA, capsid; NC, nucleocapsid; RT, reverse transcriptase; NAC, nucleic acid chaperone; PBS, primer-binding site; vRNA, viral genomic RNA; (-) SS DNA, minus strand strong-stop DNA; IP, inositol phosphate; VLPs, virus-like particles; MLV, murine leukemia virus

Retrovirus particles in which the Gag protein has not yet been cleaved by the viral protease are termed immature particles. The viral RNA within these particles shows clear evidence of the action of a nucleic acid chaperone (NAC): the genomic RNA is dimeric, and a cellular tRNA molecule is annealed, by its 3' 18 nucleotides, to a complementary stretch in the viral RNA, in preparation for priming reverse transcription in the next round of infection. It seems very likely that the NAC that has catalyzed dimerization and tRNA annealing is the NC domain of the Gag protein itself. However, neither the dimeric linkage nor the tRNA:viral RNA complex has the same structure as those in mature virus particles: thus the conformational effects of Gag within the particles are not equivalent to those of the free NC protein present in mature particles.

It is not known whether these dissimilarities reflect intrinsic differences in the NAC activities of Gag and NC, or limitations on Gag imposed by the structure of the immature particle. Analysis of the interactions of recombinant Gag proteins with nucleic acids is complicated by the fact that they result in assembly of virus-like particles. Nevertheless, the available data indicates that the affinity of Gag for nucleic acids can be considerably higher than that of free NC. This enhanced affinity may be due to contributions of the matrix domain, a positively charged region at the N-terminus of Gag; interactions of neighboring Gag molecules with each other may also increase the affinity due to cooperativity of the binding.

Recombinant HIV-1 Gag protein clearly exhibits NAC activity. In two well-studied experimental systems, Gag was more efficient than NC, as its NAC effects could be detected at a significantly lower molar ratio of protein to nucleotide than with NC. In one system, binding of nucleic acid by the matrix domain of Gag retarded the Gag-induced annealing of two RNAs; this effect could be ameliorated by the competitive binding of inositol hexakisphosphate to the matrix domain.

Correspondence to: Alan Rein; Email: reina@mail.nih.gov
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Retrovirus particles are constructed from a single structural protein, termed Gag; in fact, expression of this protein in mammalian cells is sufficient for the efficient assembly of virus-like particles in the cells. The interactions of this protein with nucleic acids are remarkably diverse, including (a) a nonspecific interaction which is essential for assembly into virus particles; (b) a specific interaction by which the genomic RNA of the virus is selectively packaged into the nascent virus particle; and (c) a nonspecific interaction in which nucleic acids are rearranged into the thermodynamically most favorable conformation. It is the latter activity which is the subject of this review.

The particles formed from retroviral Gag proteins in mammalian cells are termed "immature" retrovirus particles. These particles are converted to mature particles when the Gag protein is cleaved, in the released virus particle, into a series of fragments by the viral protease. Maturation constitutes a major change in the overall structure of the virus particle and is absolutely essential for infectivity (which is why protease inhibitors are major components of highly active antiretroviral therapy).

Gag proteins are multi-domain proteins, always containing at least three domains: matrix (MA) at their N-terminus; capsid (CA); and nucleocapsid (NC) at or near their C-terminus.[1] One major function of the MA domain is in the trafficking to and association of Gag with the plasma membrane of the virus-producing cell. CA domains are responsible for most or all of the protein-protein association between Gag molecules leading to assembly of the immature particle. Following maturation cleavage of Gag, CA molecules re-assemble within the released particle into a new structure known as the "mature core" of the virus; in HIV-1, the mature core has the architecture of a fullerene cone. Finally, the NC domain of Gag plays a principal role in the interactions of most retroviral Gag proteins with RNAs. After NC is released from Gag by PR, it participates in a wide variety of protein-RNA interactions, many involving its nucleic acid-chaperone (NAC) activity. This activity and these functions of NC are discussed in detail elsewhere in this volume. It is important to remember that interaction with nucleic acids is evidently crucial for particle formation per se: recombinant Gag proteins are soluble in vitro, but assemble into virus-like particles when nucleic acids are added.[2-4]

NAC Activity of Gag and NC in vivo

The NAC activity of free NC protein was first demonstrated over 20 years ago.[5] Among the many ways that this activity has been detected are by the ability of NC to anneal a cellular tRNA molecule to the genomic RNA of the virus (vRNA), and by its ability to promote dimerization of RNA transcripts containing sequences from the 5' untranslated region of vRNA. All retroviral vRNAs contain an 18-base sequence, the "primer binding site" (PBS), which is complementary to the 3' 18 bases of a cellular tRNA, and the appropriate tRNA is paired with the PBS in all wild-type particles. This is essential for viral replication, as the tRNA will act as the primer for DNA synthesis when the virus particle infects a new host cell.

vRNA dimerization is also of great significance for retroviral replication. The vRNAs in all wild-type retrovirus particles are dimeric: two vRNA molecules of the same (positive-sense) polarity are joined together by a limited number of base-pairs. Many lines of evidence indicate that dimerization is a prerequisite for selective encapsidation of vRNA into the assembling virus particle.[6-12] In addition, reverse transcriptase (RT) frequently switches between the two vRNA strands during DNA synthesis: this is the mechanism of genetic recombination, an important source of genetic variation in retroviruses, and may also serve as "insurance" against breaks in the individual vRNA molecules.

While NC is capable of catalyzing the dimerization of and annealing of tRNA to, vRNAs in vitro, it is clear that free NC protein does not perform these functions in vivo. This is because immature retrovirus particles, in which the Gag protein remains uncleaved because of a block in PR activity, still contain dimeric vRNAs,[7,8,13] and these vRNAs still have tRNA annealed at their PBS sites.[13-15] Both of these facts have been demonstrated for avian retroviruses (i.e., members of the alpharetrovirus genus); murine leukemia viruses (gammaretroviruses); and HIV-1 (a lentivirus). In fact, no Pol proteins (neither PR nor RT nor integrase) are required for tRNA annealing in murine leukemia virus.[16]

The fact that free NC is not required for vRNA dimerization and tRNA annealing raises the possibility that Gag, which contains NC as one of its domains, performs these functions in vivo. Alternatively, they might be catalyzed by a cellular NAC. There are several compelling arguments in favor of the idea that it is Gag, rather than an unrelated cellular protein, which is responsible. One is the fact that in alpharetroviruses and HIV-1, efficient loading of tRNA onto the PBS depends on mechanisms for concentrating the correct tRNA species into the assembling particle (reviewed in ref. 17); this suggests that the annealing event occurs within the confines of the new particle and would not be expected if a cellular protein were responsible for tRNA annealing. Second, it seems likely that the NC domain within HIV-1 Gag closely resembles free HIV-1 NC: at least on its N-terminal side, it is connected to the rest of Gag by a flexible linker.[18] Finally, recombinant HIV-1 Gag protein, purified from bacteria, is capable of catalyzing vRNA dimerization and tRNA annealing; indeed, its NAC activity seemed, in initial assays, very similar to that of NC.[19]

At the same time, the properties of immature virus particles raise the possibility that the NAC activity of Gag protein is not equivalent to that of its cleavage product, NC. Thus, the dimeric vRNAs in immature particles are less compact and less thermostable than those in mature particles.[7,8,20] There is also strong evidence that the structure of the tRNA:vRNA complex is different in immature and mature HIV-1 particles.[14,21] In the most general terms, these differences might result from differences in the NAC activities of Gag and NC; alternatively, the structure of the immature particle might constrain Gag so that the interactions catalyzed by its NAC activity cannot proceed to completion. One observation that seems to support the latter possibility is that recombinant HIV-1 Gag protein, like NC, can stabilize dimers of 345-base RNAs representing the dimer-linkage of Harvey sarcoma virus vRNAs;[19,22] as dimer stabilization normally accompanies virus maturation, this result suggests that the Gag protein has NAC capability in vitro that it does not exert within the virus particle. However, this system differs from the situation in vivo in numerous ways, and it is certainly still possible that the NAC activity of Gag is different from that of NC.

Interactions of Gag Proteins with Nucleic Acids in vitro

What are the differences between Gag and NC proteins, and how might they affect NAC activity? In addition to its NC domain, every Gag protein contains an MA domain and a CA domain. MA domains are generally basic and HIV-1 MA is capable of interacting with nucleic acids.[23-26] The MA domains of most, but not all, retroviral Gag proteins are modified at their N-termini by the 14-carbon saturated fatty acid myristic acid. (However, all the recombinant HIV-1 Gag used in the in vitro studies discussed here differs from authentic Gag in lacking the myristate modification; it also lacks the p6 domain at its extreme C-terminus). CA domains play a principal role in the interactions between Gag proteins leading to particle assembly.[27] Both of these domains might influence the binding of Gag proteins to nucleic acids, and in turn contribute to the NAC activity of the protein: MA by binding the nucleic acid substrates, and CA by introducing cooperativity into nucleic acid-binding.

Binding of HIV-1 Gag to relatively simple, short single-stranded nucleic acids has been compared with that of HIV-1 NC using fluorescence anisotropy (FA). Two studies reported that the affinities of the two proteins for the oligonucleotides were similar to each other, while a third observed a far higher affinity with Gag than with NC. Briefly, Cruceanu et al. measured binding to a 10-base oligodeoxynucleotide, $d(TG)_5$, in a buffer containing 150 mM NaCl.[28] These investigators found that both NC and Gag bound the oligonucleotide with a K_d ~ 20 nM. Jones et al. measured binding to a heteropolymeric 20-base oligodeoxynucleotide (sequence CTT CTT TGG GAG TGA ATT AG) in a buffer with 50 mM NaCl, and found that both NC and Gag exhibited K_d's ~ 30 nM (submitted for publication). However, Wu et al. tested binding to another 20-base oligodeoxynucleotide, i.e., AGC TGC TTT TTG CCT GTA CT, in a buffer with 75 mM KCl, and found that Gag bound the oligonucleotide with

a K_d of 9 nM, while that of NC was 143 nM.[29] Wu et al. and Cruceanu et al. also showed that binding of the proteins to RNA oligonucleotides was very similar to binding to the corresponding DNA oligonucleotides.

It should be noted that these affinities are really only approximations. The binding of NC to one oligonucleotide, $d(TG)_4$, has been analyzed in detail.[30] Under physiological salt conditions this interaction is remarkably complex, with more than one binding system: specifically, an NC:$d(TG)_4$ complex can bind either another oligonucleotide molecule or another NC molecule. The ability of NC to interact simultaneously with more than one nucleic acid molecule is undoubtedly one element contributing to its NAC activity.[31] Binding of Gag to short oligonucleotides has not been characterized at this level of detail, but could well be equally complex, perhaps with still more complications associated with cooperativity. (Virus particle assembly presumably involves cooperative binding of Gag molecules to nucleic acid, but this has not been documented as yet).

As several details of the experimental systems were different in all three cases, it is difficult to be sure of the reasons for the contrast in the measured affinities. However, extensive structural studies have shown that HIV-1 NC binds specifically to unpaired G residues.[32,33] Therefore, one possible explanation for the weak binding by NC observed by Wu et al. is that their oligonucleotide contained only 4 G residues.[29] Another possibility is suggested by recent observations (Goodrich, Jones and Musier-Forsyth, unpublished). They noted that in several NAC activity assays, the activity of NC was far more salt-sensitive than that of Gag. It seems possible that the hydrophobic interactions between CA domains[34-36] stabilize Gag-nucleic acid complexes, so that binding of Gag to nucleic acids is less sensitive to increases in ionic strength than that of NC. This might explain the relatively low affinity of NC measured in 75 mM KCl by Wu et al.[29] NC seems to exhibit exceptionally high affinity for the repeating sequence $(TG)_n$;[30,37,38] perhaps this explains the fact that NC bound this sequence as well as Gag, even in 150 mM NaCl.[28]

It is important to remember that experiments with very short oligonucleotides may not fully reveal the respective nucleic acid-binding properties of Gag and NC. Gag is nearly ten times the size of NC and contains two domains (MA and NC) that can bind nucleic acids; thus the footprint of Gag on a nucleic acid molecule may be significantly larger than that of NC. Further, cooperative binding, resulting from contributions of interactions between CA domains of multiple Gag molecules, will only be evident on nucleic acid molecules long enough to bind more than one Gag molecule. The binding characteristics of Gag on different lengths of nucleic acid have not been studied in any detail as yet. These measurements are in fact very difficult: as the nucleic acid becomes longer, it increases the assembly of Gag molecules into virus-like particles, so that they are removed from the solution phase.[39] However, despite the complexity of this literature, it seems likely that Gag will, in general, bind nucleic acids more tightly than free NC, provided that the nucleic acid is long enough to accommodate several Gag molecules.

Cruceanu et al. also used single-molecule DNA stretching to analyze the interactions of the proteins with long double-stranded DNA molecules.[28] This technique has previously been used by Williams and colleagues in highly illuminating studies of how HIV-1 NC (and other proteins) affect the base-paired structures of nucleic acid molecules.[40,41] Using force, rather than heat, to break base-pairs means that denaturation of the nucleic acid can be studied at physiological temperatures, where any proteins interacting with the nucleic acids remain native. These experiments showed that NC acts, in part, by weakly destabilizing base-pairs and by reducing the cooperativity of DNA denaturation, in essence promoting "breathing" of the double-stranded DNA. Another significant feature of these experiments was that there was minimal hysteresis when the force applied to the DNA was removed and the DNA was allowed to relax: this low hysteresis implies that the steady-state binding of NC to DNA is achieved by very rapid association and dissociation.

Analysis of stretching data using HIV-1 Gag indicated that the protein binds dsDNA with ~10 times higher affinity than NC.[28] It is not known whether this difference between the two proteins is due to additional interactions of the MA domain with the DNA; cooperativity induced by the CA domain; or both. It was also observed that the hysteresis when the DNA was allowed to relax in the presence of Gag was far greater than with NC; it thus appears that the off-rate for Gag is much slower than that of NC. This is particularly significant since rapid binding and unbinding appear to be essential for efficient NAC activity.[28,31]

NAC Activity of HIV-1 Gag Protein

Wu et al.[29] and Jones et al. (submitted for publication) both performed detailed analyses on the NAC activity of HIV-1 Gag. The study by Wu et al. compared Gag with NC in their effects on reverse transcription in a reconstituted minus-strand transfer system. This system recapitulates in vitro an early step in viral DNA synthesis. In vivo, the initial DNA product, termed "minus strand strong-stop DNA" or (-) SS DNA, is 181 nucleotides long and is complementary to the R and U5 regions at the extreme 5' end of the vRNA. Before the remainder of the vRNA can be copied, the R portion of (-) SS DNA must anneal to the complementary R sequence at the 3' end of vRNA.

This annealing event normally depends upon the NAC activity of NC; the NAC requirement is particularly stringent because the 3' end of (-) SS DNA, like the sequences in the vRNA to which it must anneal, contains a stable, intramolecularly base-paired structure, called TAR in the vRNA. Normally, NC blocks a dead-end reverse transcription reaction called "self-priming", in which the (-) SS DNA folds back on itself and the resulting hairpin is extended by RT; instead, the NAC activity of NC promotes strand transfer, i.e., the proper annealing of (-) SS DNA to a complementary "acceptor RNA".[42] Wu et al.[29] detected this annealing event by measuring the extension of the (-) SS DNA when RT copies the acceptor RNA all the way to its 5' end. The strand-transfer step has previously been analyzed in detail; while it is promoted in vivo by NC, Wu et al. used it as an experimental system with which to explore the NAC capabilities of Gag.

Wu et al.[29] found that Gag was more efficient than NC in their assay, in the sense that it could promote strand transfer at

significantly lower concentrations. However, they also observed that this effect was inhibited by higher levels of Gag: strand-transfer reached a maximum level at 0.12 μM Gag, but was reduced to background levels in 0.46 μM Gag. In contrast, strand-transfer increased monotonically as NC was raised from 0.4 to 3.2 μM. The mechanism responsible for inhibition of strand transfer at higher Gag concentrations is discussed briefly below.

Wu et al. also performed a number of experiments which helped to shed light on the reasons underlying the differences between the NAC activities of Gag and NC. First, they found that fragments of Gag lacking NC were completely unable to promote strand transfer. These fragments also had no significant effect in trans on the strand-transfer activity of NC. Thus, the NC domain is absolutely required for the NAC activity of Gag and appears to be the domain responsible for this activity.

They also tested Gag proteins lacking part or all of the MA domain. The activities of these proteins were similar, but somewhat lower, than that of Gag. Surprisingly, the protein lacking residues 16 through 99 in the MA domain was less active than the protein lacking the entire MA domain; the reason for this is not clear.

Finally, Wu et al. made the important observation that annealing per se of the (-) SS DNA with its complementary RNA, unlike the promotion of strand transfer, is not inhibited by supraoptimal concentrations of Gag. Therefore, the inhibition observed in the strand-transfer reactions is really a specific inhibition of DNA synthesis. This may reflect the slow dissociation of Gag from single-stranded nucleic acid noted above; in other words, once Gag binds RNA, it may act as a "roadblock" for RT. It may also be related to the fact that (at least at higher protein-NA concentrations) addition of nucleic acid can remove both Gag and nucleic acid from solution by inducing assembly of virus-like particles.[2]

Somewhat analogous observations were also reported by Roldan et al.[43] These investigators compared NC with "mGag", a bacterially produced protein containing the C-terminal domain of CA and SP1 (the "spacer" between CA and NC) as well as NC (and 36 nonviral residues at its N-terminus). They analyzed the effects of these proteins on the annealing of primer tRNA to vRNA and on the extension of the tRNA primer by RT. Like Wu et al.[29] they found that mGag appeared to have a higher affinity for the RNAs and annealed the RNAs more efficiently than NC, but that high concentrations of mGag inhibited DNA synthesis.

The second situation in which the NAC activity of HIV-1 Gag has been carefully compared with that of NC is in a system modeling the annealing of tRNA to the PBS. As noted above, this annealing event is an authentic function of the Gag protein (reviewed in ref. 17). Jones et al. (submitted) found that, just as with the strand-transfer reaction discussed above, Gag is more efficient at catalyzing tRNA annealing than NC: one Gag molecule per ~30 nt was sufficient for 50% annealing in a 2 hr reaction, while for NC one molecule per ~8 nt was required. One possible explanation for this difference could be that, as noted above, a single Gag molecule can bind a larger stretch of nucleic acid than a single NC molecule; this has not been investigated. However, while the efficiency of annealing by Gag was superior to that of NC, the rate at which Gag anneals tRNA was over 10 times lower than that catalyzed by NC. Fragments of Gag lacking the NC domain were completely incapable of catalyzing annealing, suggesting again that the NC domain is responsible for the NAC activity of Gag.

Analysis of a series of Gag fragments also showed that the relative slowness of annealing by Gag depends upon the presence of an intact MA domain. The role of MA was also demonstrated in experiments (described below) with inositol hexakisphosphate (IP6). Previous studies have shown that when recombinant HIV-1 Gag protein is mixed with single-stranded nucleic acid, it assembles into very small spherical virus-like particles (VLPs).[2] These VLPs are only 25–30 nm in diameter, whereas an authentic immature particle is ~100 nm in diameter. However, when the nucleic acid is supplemented with IP5, VLPs of the correct size are formed.[44] Several lines of evidence indicate that the MA domain of Gag is essential for its interactions with IP5 and IP6;[34,44,45] in fact, these IP's resemble the headgroups of phosphoinositides present in the plasma membrane, and binding of the MA domain of HIV-1 Gag to phosphatidylinositol (4,5) bisphosphate is evidently critical for plasma membrane targeting.[46,47]

How might IP's influence VLP assembly? The Gag molecules in an authentic immature particle are radially arranged rods ~20 nm long.[48-50] As rods of this size could not fit within 25–30 nm VLPs, the Gag molecules making up these small VLPs must be bent. (HIV-1 Gag protein in free solution is also bent[51]). Presumably, both MA and NC domains interact with nucleic acid in the small VLPs assembled in nucleic acid alone, but when IP5 is also present, it can bind the MA domain while NC remains bound to nucleic acid. Under these conditions the protein can extend into a rod and assemble into full-size VLPs.[44] In other words, IP's can compete with nucleic acid for sites in the MA domain; dissociation of the MA domain from nucleic acid enables Gag to extend into a rod.

A similar mechanism appears to be at work in tRNA annealing to the PBS. Jones et al. made the remarkable observation that the annealing catalyzed by Gag is accelerated over 10-fold by addition of IP6 or other IP's to the reaction. However, the IP's had no effect on annealing induced by NC, CA-NC or even full-length Gag in which lysines 30 and 32, known to be crucial for IP binding by the MA domain, were replaced by asparagines. Taken together, these results strongly support the idea that both the MA and NC domains of full-length Gag can bind nucleic acids, and that binding by the MA domain retards tRNA annealing. IP's abrogate this retardation by competing for the MA domain, allowing the unfettered NAC action of the NC domain. This model is depicted in **Figure 1**. It is intriguing to note that in HIV-1 Gag, the membrane-binding and nucleic acid-binding activities of the MA domain may reciprocally influence each other: while the work of Jones et al. suggests that the NAC activity of Gag is inhibited unless the MA domain binds to a lipid headgroup, that of Chukkapalli et al.[24] implies that interaction with RNA modulates the membrane-binding properties of the MA domain. This interaction apparently results in the specific targeting of Gag to the plasma membrane, but not other cellular membranes.

We have recently characterized the solution properties of the Gag protein of Moloney murine leukemia virus (MLV). We

found (Datta et al., manuscript in preparation) that MLV Gag is an extended rod in solution, with approximately the same shape and dimensions as it has in immature virus particles. As discussed above, Jones et al. have shown that in HIV-1 Gag, which is bent in solution,[51] the MA domain has a major effect on the NAC activity of the protein; it will be of great interest to learn whether this is true for MLV Gag as well.

In conclusion, it is clear that the NAC activity of Gag is important during the initial assembly of immature retrovirus particles. In general, Gag is more efficient than free NC in in vitro assays of NAC activity. However, in one experimental system, modeling the annealing of tRNA to the PBS, annealing by saturating levels of Gag is significantly slower than annealing by NC; this is apparently due to interference by the MA domain with the NAC action of the NC domain. We do not know why this phenomenon has only been observed in this assay. Finally, despite the apparent superiority of Gag to NC in several in vitro assays, the dimerization of vRNA and annealing of tRNA to the PBS both appear to be incomplete in immature virus particles. This may reflect structural constraints within the virion.

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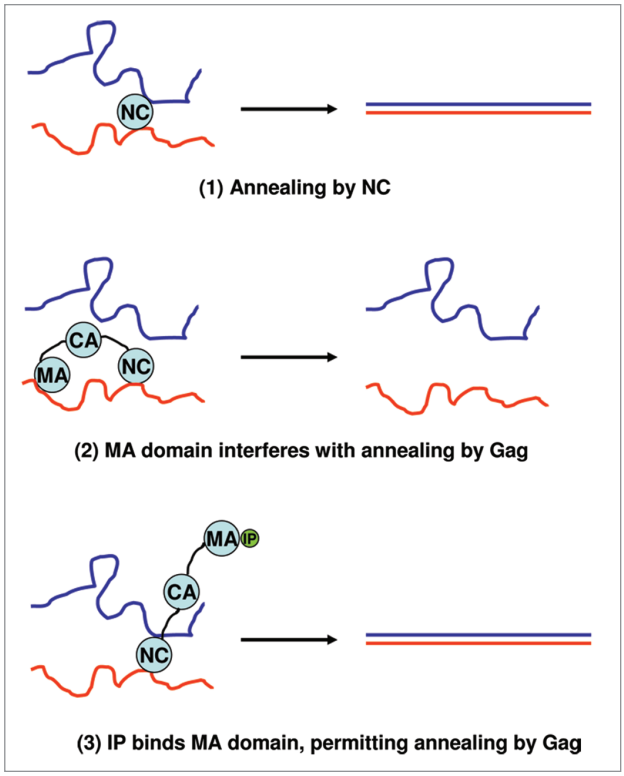

Figure 1. Role of inositol phosphates (IP) in relieving MA inhibition of Gag NAC action, as described by Jones et al. (1) Annealing of complementary nucleic acid molecules by free NC protein; (2) Inhibition by the MA domain of annealing of complementary nucleic acids by Gag; (3) Relief of the inhibition by addition of an inositol phosphate, which binds the MA domain.

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