Structural order in Pannexin 1 cytoplasmic domains

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Abbreviations: Panx1, pannexin 1; Panx2, pannexin 2; Panx3, pannexin 3; Cx43, connexin 43; CD, circular dichroism; NMR, nuclear magnetic resonance; TM, transmembrane; NT, N-terminus; CL, cytoplasmic loop; CT, C-terminus; TFE, 2,2,2-trifluoroethanol

Pannexin 1 forms ion and metabolite permeable hexameric channels with abundant expression in the central nervous system and elsewhere. Although pannexin 1 does not form intercellular channels, a common channel topology and oligomerization state, as well as involvement of the intracellular carboxyl terminal (CT) domain in channel gating, is shared with connexins. In this study, we characterized the secondary structure of the mouse pannexin 1 cytoplasmic domains to complement structural studies of the transmembrane segments and compare with similar domains from connexins. A combination of structural prediction tools and circular dichroism revealed that, unlike connexins (predominately intrinsically disordered), cytosolic regions of pannexin 1 contain approximately 50% secondary structure, a majority being α-helical. Moreover, prediction of transmembrane domains uncovered a potential membrane interacting region (I360–G370) located upstream of the caspase cleavage site (D375–D378) within the pannexin 1 CT domain. The α-helical content of a peptide containing these domains (G357–S384) increased in the presence of detergent micelles providing evidence of membrane association. We also purified a pannexin 1 CT construct containing the caspase cleavage site (M374–C426), assigned the resonances by NMR, and confirmed cleavage by Caspase-3 in vitro. On the basis of these structural studies of the cytoplasmic domains of pannexin 1, we propose a mechanism for the opening of pannexin 1 channels upon apoptosis, involving structural changes within the CT domain.

The core proteins of chordate gap junctions are connexins and of invertebrates are innexins. Although connexin homologs have not been found in invertebrates, a BLAST search for innexin homologs in chordate genomes identified the pannexin gene family.1 The family has 3 members, pannexin 1 (Panx1), pannexin 2 (Panx2), and pannexin 3 (Panx3). Panx1 is the most ubiquitously expressed and the most widely studied, particularly with respect to functional properties.

There is no sequence homology between pannexins and connexins, although there is topological similarity, both families encoding proteins with four transmembrane domains and both carboxyl and amino termini within the cytoplasm. Similarly to connexins, Panx1 oligomerizes as a hexamer and traffics to the plasma membrane to form channel-like structures, as visualized with electron microscopy.2,3 Unlike connexins, which connect cell interiors without access to extracellular space, Panx1 channels are non-junctional, allowing movement of ions and small molecules between inside and outside of cells.4–6 Diameter of the Panx1 pore was estimated to be between ~17 and 21 Å, which is significantly larger than the well-studied 43 kDa connexin (Cx43), ~15 Å, consistent with early electron micrographs showing larger particles in invertebrate than vertebrate gap junctions.3,7,8

Other similarities between connexins and Panx1 are the involvement of the carboxyl terminus in opening or closing the channel and the potential for phosphorylation of cytoplasmic serine, threonine or tyrosine residues to modify such gating. For Cx43, channel closure in response to acidification and transjunctional voltage gradients has been attributed to binding of carboxyl terminus (CT) to the cytoplasmic loop (CL) domain.9,10 In the case of pH gating, channel closure appears to result from acquisition of α-helicity within cytoplasmic Cx43 domains, giving rise to increased inter-domain affinity.11 Phosphorylation of a specific serine residue on the cytoplasmic CT of Cx43 (S365) also changes the structure of the region, inhibiting phosphorylation of adjacent residues that would otherwise induce channel closure.12 Inter-domain interactions are also hypothesized to underlie gating of Panx1. Release of ATP has been attributed to caspase cleavage of the CT of Panx1 at residue D378,13 and the co-expression of hPanx1ICT299–426 or hPanx1ICT299–391 with the constitutively active truncated hPanx1 Δ371 restored the basal inhibition of the channel.13,14 Phosphorylation of residues in the cytoplasmic domains of Panx1 is predicted from its amino acid sequence.15,16 Src family kinases have been shown to be involved in rPanx1 channel activation in CA1 neurons in response to anoxia, suggesting the phosphorylation of tyrosine(s), although
phosphorylation of the putative Src consensus site was not demonstrated. P2X7 receptor activation of Panx1 channels in J774 cells was also suspected to involve a Src tyrosine kinase, but likewise, Panx1 tyrosine phosphorylation was not detected. More recently, during skeletal muscle potentiation, release of ATP through Panx1 channels was shown to be accompanied by increased serine and threonine phosphorylation, although specific phosphorylated residues were not identified.

Altogether, these data demonstrate the importance of Panx1 cytoplasmic domains and the need to further elucidate the mechanism of pore closure. In this study, we used a combination of structural prediction tools and circular dichroism (CD) to characterize the cytosolic domains of mPanx1. Unlike the cytoplasmic domains of connexins that are mainly intrinsically disordered, significant portions of the mPanx1 N-terminus (NT), CL, and CT adopt diverse secondary structures. We also provide evidence that a potential membrane interacting region located within the mPanx1 CT interacts with lipids and this association affects secondary structure. Finally, NMR was used to verify that Caspase-3 can interact and cleave the mPanx1 CT in vitro. These data provide the first structural information of the cytoplasmic domains of mPanx1, groundwork that will allow further clarification of the function and regulation of Panx1 channels.

Results

Structural prediction of mPanx1

To assign cytoplasmic and transmembrane (TM) domains of mPanx1, four TM prediction programs based on distinct algorithms were used (Fig. 1A): (1) ΔG prediction server v1.0, which uses the apparent free energy difference necessary for insertion into a membrane; (2) TMpred program, which utilizes the statistical analysis of the sequence against Tmbase (database of naturally occurring TM proteins); (3) Dense Alignment Surface (DAS), which calculates hydrophobicity profiles based on low-stringency dot-plots of the sequence against a library; and (4) TopPred program, which generates a hydrophobicity profile. All algorithms predicted four TM domains, with
A mPanx1 NT

| Sequence | MAIHALETYFVDLLEKPEPPFKGLLAVD |
|----------|--------------------------------|
| GOR4     | H                     H         |
| Jpred    | H                     H         |
| Predict  | H                     H         |
| PSIPRED  | H                     H         |

**B mPanx1 CL**

| Sequence | FSAPRFLCDELVBMELYVRNNLAAMKSGRDGPGDFPVTENQSLWNIDERHPYTPTPTEQLATKNSNHILMYALL |
|----------|-------------------------------------------------------------|
| GOR4     | H                     H                     H           |
| Jpred    | H                     H                     H           |
| Predict  | H                     H                     H           |
| PSIPRED  | H                     H                     H           |

**C mPanx1 CT**

| Sequence | GCDILFVCTILTFVLYLSKEDLGYLYLGLSHSIEKLCYLVELENKSPQIDMLL179GKNDTIDTIK179QKGEQDGQVRSFYDGDL6L56EAAANWGEKNSRQLLLFSC |
|----------|-------------------------------------------------------------|
| GOR4     | H                     H                     H           |
| Jpred    | H                     H                     H           |
| Predict  | H                     H                     H           |
| PSIPRED  | H                     H                     H           |

Figure 2. Secondary structure prediction of the mPanx1 cytoplasmic domains. Display of the predicted secondary structure of (A) mPanx1 NT, (B) mPanx1 CL, and (C) mPanx1 CT obtained by utilizing four different prediction algorithms: GOR4, Jpred, Predict (PROF), and PSIPRED. Prediction per residue is labeled under the sequence as follow: H, α-helix; E, β-strand; black dash, random coil. Peptides used for the rest of the study are indicated under the sequences. The segment expected to interact with the membrane and the caspase cleavage site are respectively indicated with a wavy and solid black line.

limits similar to previously described studies: TM1, 37–56; TM2, 107–127; TM3, 217–235; TM4, 277–296. A unique domain, however, composed of about 10 mainly hydrophobic residues in the CT domain (~I360-G370) also emerged from all 4 algorithms (indicated with black arrows in Fig. 1A). The prediction values did not meet the criteria for an actual TM domain (below cutoff) but suggests a potential membrane interacting region. A similar domain was also identified in the hPanx1, which shares 86.2% identity with mPanx1, indicating a similar feature across species (data not shown).

To investigate the secondary structure of the mPanx1 cytoplasmic domains, the primary sequence of the NT, CL, and CT (delineated by the TM prediction calculations) were submitted to 4 different secondary structure prediction algorithms: (1) GOR4, based on information theory; (2) Jpred and (3) Predict (PROF), both established from database set comparison; and (4) PSIPRED, obtained by analysis of output from PSI-BLAST (Fig. 2). All 4 sets of predictions largely overlapped. Surprisingly, unlike connexins whose cytoplasmic domains are mainly intrinsically disordered, mPanx1 counterparts are predicted to present an assortment of α-helices linked by random coiled segments and some β-strands, especially in the mPanx1 NT and CT domains (Fig. 2A and C). It is noteworthy that the secondary structure prediction for the suspected membrane interacting domain (~I360-G370) is mainly α-helical, followed by β-strand structure in the putative caspase cleavage site. Based on the secondary structure prediction consensus, we designed peptides of 14 to 33 amino acids to preserve the integrity of domains with predicted structure and to validate their structural predictions by CD. Peptides used for the rest of the study are indicated under the sequences in Figure 2.

Secondary structure studies of mPanx1 cytoplasmic domain peptides by CD

Peptides are commonly used to study the secondary structure of proteins; however, caution is needed when interpreting implications for domain structure of membrane proteins as peptides lack long-range constraints that may impact their folding (e.g., being tethered to a TM domain). Therefore, the co-solvent, 2, 2, 2-trifluoroethanol (TFE) was used as a tool to probe the secondary structure propensity of the mPanx1 peptides. Although TFE is primarily known as an α-helix-stabilizing co-solvent, some studies have also shown efficiency of TFE to stabilize β-strands. CD spectra were collected for each peptide in the absence and presence of increasing amount of TFE, however only up to 30% to ascertain intrinsic secondary structure without inducing any non-native conformation (Fig. 3). As a general indication, CD spectra of α-helices present double minima at 208 nm and 222 nm and a positive peak at 193 nm; β-strands show a minimum at 217 nm and positive peak at 195 nm, and intrinsically disordered peptides are marked by a minimum near 195 nm and very low ellipticity above 210 nm.

Overall, most of the CD findings were consistent with the secondary structure predictions. The C-terminus of the mPanx1 CT (CT4 and CT5) was mainly random coiled as was the middle of the mPanx1 CL (CL2) (spectra presenting minimum at 198 nm with only a weak peak at 222 nm). The addition of
30% TFE only slightly increased the amount of α-helical structure in these peptides. mPanx1 NT1 and CT1 showed spectra consistent with random coiled structure; however, they have a propensity to form an α-helix at 15% TFE. Similarly, mPanx1 CL3 and CT2 were mainly random coiled in absence of TFE but exhibited α-helical structure with as little as 5% TFE, suggesting a higher propensity to form an α-helix than the mPanx1 NT1 and CT1 domains. Finally, the C-terminus of the NT and the N-terminus of the CL were inherently structured in absence of TFE. NT2 presented a spectrum characteristic of a β-strand with a minimum at 217 nm, while the CD spectrum of CL1 exhibited 2 minima at 208 and 222 nm typical of an α-helix. Increasing the amount of TFE did not impact the structure of these peptides. Interestingly, the mPanx1 CT3 encompassing the membrane interacting domain (~I360-G370) and caspase cleavage site (D375-D378) displayed a spectrum suggesting a mixture of α-helix and β-strand, consistent with the secondary structure prediction (Fig. 2C). In presence of 30% of TFE though, the peptide preferred an α-helical structure as shown by the spectrum with a double minima around 208 and 220 nm. In summary, out of the 10 peptides analyzed, 5 of them were disordered or presented a low propensity to form secondary structures (NT1, CL2, CT1, CT4, and CT5), 2 showed a very high propensity to form α-helices (CL3 and CT2), and 3 of them were inherently structured (α-helix and/or β-strand; NT2, CL1, and CT3). Significantly, unlike the predominantly unstructured nature of

![Figure 3. Circular Dichroism (CD) analysis of mPanx1 peptides. CD spectra of (A) mPanx1 NT1 and NT2, (B) mPanx1 CL1, CL2, and CL3, (C) mPanx1 CT1, CT2, CT3, CT4, and CT5 in 1x PBS in the presence of 0 (solid line), 5 (dashed line), 15 (dotted line) and 30% (dashed and dotted line) TFE (2,2,2-trifluoroethanol).](image-url)
most connexin intracellular domains, about 50% of the cytoplasmic residues of mPanx1 are expected to be involved in some sort of secondary structure in their native state.

Secondary structure change of mPanx1 CT3 in presence of lipid

The TM prediction programs suggested that the mPanx1 CT segment comprising residues I360-G370 interacts with the membrane. To test this possibility, we collected CD spectra for the mPanx1 CT3 peptide in the presence and absence of 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG), a membrane mimetic detergent (Fig. 4). Upon addition of 8% LPPG, the spectrum exhibited a second minimum at 222 nm and an increased positive maximum at 190 nm, indicating an increase in α-helix structure. Interestingly, CD revealed that the CT3 peptide alone (no LPPG) contained a mixture of α-helix and β-sheet structure; however, an α-helical conformation was more prominent in presence of 30% TFE. Altogether, the data support the possibility that the central portion of the mPanx1 CT domain interacts with the membrane, and the association increases the α-helical content of this domain.

Assignment of resonances for amino acids comprising the mPanx1 CT374–426

To provide a global perspective to complement the peptide CD data, NMR was used to investigate the secondary structure on a residue-by-residue basis as well as determine the location of molecular partner interactions. We initially focused on the mPanx1 CT because of the unique membrane interacting domain (-I360-G370), reported cleavage by Caspase-3, and the presence of predicted sites of phosphorylation. The full-length mPanx1 CT domain (Q300-C426) was insoluble. While it is conceivable that solubility might have been improved if reconstituted in a lipid environment, we attempted to bypass this problem by cloning 2 separate mPanx1 CT domains (mPanx1 CT300–356 and CT374–426), thereby excluding the hydrophobic segment. Unfortunately, although both polypeptides overexpressed, only the mPanx1 CT374–426 was present in the soluble fraction after cell lysis (data not shown). Therefore, mPanx1 CT374–426 was purified in 1x PBS (pH 7.4) to a final concentration of 3 mM. All 1H, 13N, 13C backbone resonances and most side chains were assigned at 7 °C (BioMag ResBank database, accession number 19814). Data were collected at a low temperature (7 °C vs. 25 °C) to reduce the molecular tumbling and increase the spectral resolution. Presented in Figure 5A is the assigned 15N-HSQC spectrum of the mPanx1 CT374–426 domain. The 15N-HSQC spectrum, in which each amino acid (except Pro) gives a discrete signal, displays a narrow chemical shift dispersion in the 1H dimension typical of intrinsically disordered domain. This observation is in agreement with CD data and analysis of the chemical shifts. CD shows the mPanx1 CT374–426 is predominantly unstructured even in the presence of increasing concentration of TFE (Fig. 5B). Meanwhile, evaluation of the chemical shift deviations with respect to the random coil value (compared with the Wishart database), which is used to predict secondary structure, indicates the lack thereof for the mPanx1 CT374–426 domain (Fig. 5C).

In vitro Caspase-3 cleavage assay of mPanx1 CT374–426

When studying a soluble domain from a membrane protein, a critical question is whether it retains the same biological function as when in the native condition. Therefore, the ability was assessed of the mPanx1 CT374–426 construct to be cleaved by the known protein partner, Caspase-3.13 Active recombinant Caspase-3 (25U) was added to purified labeled mPanx1 CT374–426, and the cleavage reaction was monitored by NMR. After 1 h incubation at 37 °C, a decrease of intensity of the peaks surrounding the caspase cleavage site (I376-I381) as well as the appearance of 4 new peaks was observed (Fig. 6, new peaks indicated with black arrows). An additional 25U of Caspase-3 followed by 2 more hours at 37 °C led to complete cleavage of the mPanx1 CT374–426 construct after residue D378. This is evident by the 3 residues on each side of the cleavage site disappearing congruent with an increase in the intensity of the 4 new peaks. Interestingly, 3 of the 4 new peaks presented chemical shift close to the 3 residues preceding the cleavage site, I376, I377, and D378, suggesting that they represent the chemical shift of these 3 residues in the GPLGSMD11 peptide released upon cleavage (Fig. 6, inset). Meanwhile, the signals of G379, K380, and I381 residues, localized on the C-terminal of the cleavage site disappeared and only one of them, most likely I381, emerged as a new peak. This is not surprising since G379 and K380 constitute the new N-terminal of the cleaved peptide G379-C426, and the dynamic nature of the N-terminal residues of a soluble protein usually prevents their detection in the 15N-HSQC spectrum.

Discussion

Here we provide the first structural information regarding the cytoplasmic domains of mPanx1. These studies complement the data of rPanx1 obtained by electron microscopy that defined the general architecture of the channel and the pore, but were unable to give insight regarding the soluble domains.2,5 CD and secondary structural prediction data revealed that the mPanx1 cytosolic domains present a significant amount of secondary structures,
estimated at up to 50% of the primary sequence. mPanx1 regions
NT2, CL1, and CT3 were inherently structured, respectively
β-strand, α-helix and a mixture of both β-strand and α-helix,
and the CL3 and CT2 domains displayed a high propensity for
α-helix conformation (Fig. 7). Our laboratory has also per-
formed similar structural studies with several connexin cytoplas-
mic domains and while they are predominately disordered, they
do contain small domains with inherent propensity and suscepti-
bility to adopt secondary structure. Interestingly, the struc-
tured segments of mPanx1 and some of the connexins somewhat
overlap when comparing their cytoplasmic domains and proposed
distance from the membrane. For example, the second half of the
CL domains of Cx43, Cx36 and Cx32 exhibit a high propensity to
form α-helical structure, similarly to mPanx1 CL3. Meanwhile,
there is a correlation between the centrally located α-helices of the
mPanx1, Cx40, Cx43, and Cx45 CT domains.

Secondary structural predictions for the cytosolic domains
of mPanx2 and mPanx3 were also calculated employing the algo-
rithms used for mPanx1 (Fig. 8). Similarly to mPanx1, mPanx3
NT contains some β-strand structure in its second half, while
mPanx2 NT is mainly α-helical. The most similarities are found
in the CL domains with α-helices linked by random coiled por-
tions. Finally, like the mPanx1 CT, the mPanx3 CT presents a
central α-helical segment and the second half of Panx2 CT is
mainly intrinsically disordered. Noticeably, the mPanx2 CT
shows only a structural consensus for three small α-helices in its
first half.

Another interesting feature detected by TM prediction
algorithms is a potential membrane interacting segment span-
nning I360-G370 in the mPanx1 CT; residues that present high
hydrophobicity and α-helical content. Here, we provide the
first evidence that this mPanx1 CT domain could interact with
the membrane as its secondary structure composition was modi-
fied (i.e., increase in α-helical content) in presence of mem-
brane mimetic lipids. mPanx2 and mPanx3 sequences were also
subjected to the same TM prediction servers (Fig. 1B and C).
There was no consensus for such a membrane interacting
domain in the CT of mPanx2; however, the results for mPanx3
converged to identify a segment of ~20 residues modeled as
a fifth TM domain. The secondary structure of this region,
like mPanx1, is predicted to be α-helical (Fig. 8B). Although
the definitive topology of mPanx3 remains to be clarified, the
existence of a real fifth TM is less likely and this domain is
expected to interact with the membrane similarly to mPanx1
CT I360–G370. A “reentrant” loop, or half-membrane spanning
domain located between 2 TM domains is commonly found
in ion channels, such as the Kcsa potassium ion channel and
the glutamate receptor ion channel. However, to our knowl-
edge, a membrane interacting domain localized in the cytosolic
C-terminal of an integral protein is less frequent. Moreover, the

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Figure 5. Structural study of the mPanx1 CT374–426 domain. (A) 15N-HSQC spectrum of the mPanx1 CT374–426. Each resonance peak has been labeled. (B) CD spectra of mPanx1 CT374–426 in 1x PBS in the presence of 0 (solid line), 5 (dashed line), 15 (dotted line) and 30% (dashed and dotted line) TFE. (C) Chemical shift index analysis for 1Hα, 13Cα, 13C, and 15N obtained by comparison to the Wishart database.
location of this segment is of importance as cleavage of residues situated just downstream by Caspase-3 and -7 (after D378) is a mechanism to open Panx1 channels to release ATP into the extracellular space during apoptosis.\textsuperscript{13,43,44}

While generally found in a closed state, in addition to apoptosis, Panx1 channels can become active (high conductance pores that are non-selectively permeable to ions and small molecules of up to 1.5 kDa) by mechanical stimulation, elevation of
extracellular [K+] or through purinergic receptors (for review see ref. 45). The exact molecular details that maintain Panx1 channels in the closed state are still not clear, but a “particle-receptor” model between the CT and the pore, comparable to connexins, has been suggested. CT residues involved in this mechanism have emerged from studies involving hPanx1 deletion constructs and peptides. The hPanx1Δ371 construct is constitutionally active while hPanx1Δ391 resembles that of the wild type.13,14 Additionally, the hPanx1 peptide CT380–391 had no inhibitory effect on activated hPanx1 channels, as opposed to the co-expression of hPanx1 CT299–391 that closed the pore.14 Together, these data indicate involvement of residues I372–D379 in pore closure. Of note, these residues overlap with the caspase cleavage site and are in close proximity to the membrane interacting domain.

Based upon the structural analysis provided herein and data from the literature, we propose a model where the mPanx1 CT372–379 domain participates in channel gating: under normal physiological conditions (closed state), association of residues I360-G370 with the membrane enables residues 372–379 to interact with the pore forming domain as an α-helix. Mechanical stimulation, apoptosis, elevation of extracellular [K+], and purinergic receptors cause, by yet to be determined mechanism(s), release of residues 372–379 from the pore forming domain. Residues 372–379 then switch secondary structure to a β-strand. In the case of apoptosis, this forms a high affinity binding site for caspases to facilitate cleavage. This succession of events, helped by the tethering of this domain to the membrane interacting segment, leads to the activation of the channel and the release of ATP outside of the cell. Further investigation will have to be performed to confirm or refute this model.

Materials and Methods

Peptide synthesis, and expression and purification of Panx1 CT374–426

Panx1 peptides described in Figure 2 and 3 were synthesized by LifeTein (95% purity). The mPanx1 CT374–426 polypeptide was subcloned into the bacterial expression vector pGEX-6P-2 (GST-tagged; http://www.gelifesciences.com/catalogue/item/28-9546-50) by standard PCR methods and transformed for overexpression in BL21(DE3) competent cells (http://www.genomics.agilent.com/catalogue/item/200131). Growth and purification of the 15N13C-labeled polypeptide was conducted as previously described for recombinant GST-tagged protein, the tag being removed by Turbo3C protease digestion (http://www.accelagen.com/catalog/item/H0101S).10,11,46

Circular Dichroism (CD)

CD experiments were performed on a JASCO J-815 CD spectrometer at 25 °C in the far UV (260–190 nm) with a 0.1 mm path length quartz cell, using a bandwidth of 1 nm, an integration time of 1 s, and a scan rate of 50 nm/min. Each spectrum is the average of 5 scans. All spectra were corrected by
subtracting the solvent spectrum acquired under identical conditions. The synthetic or recombinant peptides were in solution in 1x PBS, in the presence of various amount of TFE or 1-palmitoyl-2-hydroxy-sn-glycero-3-[phospho-RAC-(1-glycerol)] (LPPG), at final concentrations between 0.3–0.9 mM depending on the peptide CD signal. All CD data were processed and converted to mean residue ellipticity to account for the peptide size and concentration differences using Spectra Analysis from the Jasco Spectra Manager software, Version 2.05.01.

Nuclear Magnetic Resonance (NMR)

NMR data were acquired at 7 °C using a 600 MHz Varian INOVA NMR spectrometer outfitted with a cryo-probe. Purified 15N13C-labeled Panx1 CT374-426 (0.1 mM in 1x PBS, 10 mM DTT, and 5 mM EDTA) was incubated at 37 °C for 1 hin presence of 25U of recombinant Caspase-3 (http://www.enzolifesciences.com/catalogue/item/ALX-201-059). To bring the cleavage to completion, an additional 25U of Caspase-3 was added followed by incubation for 2 more hours at 37 °C. Progression of the reaction was followed by collecting 15N-HSQC experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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