

Trafficking and surface expression of the glutamate receptor subunit, KA2

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Abstract

Kainate receptors are a class of ionotropic glutamate receptors that are widely expressed in the mammalian brain, yet little is known about their physiological role or the mechanisms by which they are regulated. Kainate receptors are composed of multiple subunits (GluR5–7; KA1–2), which can combine to form homomeric or heteromeric channels. While the kainate receptor subunit KA2 can combine with GluR5–7 to form heteromeric channels, it does not form functional homomeric channels when expressed alone. In an attempt to identify the molecular mechanisms for this, we have characterized the trafficking and surface expression of KA2. We find that KA2 alone does not traffic to the plasma membrane and is retained in the endoplasmic reticulum (ER). In contrast, co-expression with GluR6 disrupts ER-retention of KA2 and allows plasma membrane expression. Using a chimeric reporter protein we have identified an ER-retention motif within the KA2 cytosolic domain. Recent studies have identified a consensus ER-retention motif (RRR) that is contained within both the NMDA receptor NR1 subunit and K⁺ channels. While KA2 contains a similar stretch of amino acids within its C-terminus (RRRRR), unlike the NR1 motif, disruption of this motif with alternating glutamic acid residues does not disrupt ER-retention of KA2, suggesting a unique mechanism regulating KA2 surface expression.

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Fast excitatory synaptic transmission in the central nervous system (CNS) is mediated predominantly by the ionotropic glutamate receptors which can be divided into three classes: α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), *N*-methyl-D-aspartate (NMDA), and kainate receptors [1,2]. Although AMPA and NMDA receptors have been extensively studied, kainate receptors have received less attention primarily due to the lack, until recently, of pharmacological agents that distinguish between AMPA and kainate receptors [3,4]. Kainate receptors are made up of different combinations of the GluR5–7 and KA1 and 2 subunits, which are widely expressed throughout the CNS [2]. Kainate receptors participate in synaptic transmission

both pre- and post-synaptically [4–9], have roles in regulating both excitatory and inhibitory synaptic transmission [4,9], and are involved in long-term potentiation at hippocampal mossy fiber-CA3 synapses [10,11] and at developing thalamocortical synapses [12].

Many oligomeric protein complexes contain subunits that are only expressed at the plasma membrane when assembled in the appropriate subunit stoichiometry. In many cases, the fidelity of oligomerization is maintained by retaining unassembled protein subunits in the ER [13]. Several ER-retention motifs have been described that are encoded within transmembrane proteins, including the classic di-lysine (KK) and di-arginine (RR) motifs. Recently, a variation of this positively charged motif has been identified in multi-spanning transmembrane domain proteins such as the inwardly rectifying K⁺ channels and NMDA receptors (RXR; for review,

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see [14,15]). The NMDA receptor subunit NR1 contains a stretch of three arginines (RRR) that regulate ER-retention and disruption of this motif results in plasma membrane expression [16–18].

The NMDA receptor subunit NR1 does not form functional homomeric channels when expressed alone in mammalian cells. Similarly, the kainate receptor subunit KA2 does not form functional channels unless co-expressed with another kainate receptor subunit (GluR5–7), leading to the possibility that the formation of an oligomeric receptor complex masks an ER-retention motif in KA2. In the current study, we investigated the trafficking and surface expression of KA2 to examine the mechanisms regulating plasma membrane expression of KA2. We find that KA2, unlike GluR6, does not traffic to the plasma membrane when expressed in heterologous cells, but instead is ER-retained. However, upon co-expression with GluR6, KA2 traffics to the plasma membrane and forms functional heteromeric kainate receptors. The addition of the KA2 C-terminus to the plasma membrane protein Tac results in ER-retention of the TacKA2 chimera, thereby revealing the presence of a dominant ER-retention motif in the KA2 C-terminus. Like NR1, KA2 contains a positively charged motif (RRRRR) within its C-terminus. However, unlike NR1, disruption of the RRRRR motif with alternating glutamic acid residues does not result in plasma membrane expression, demonstrating a unique mechanism regulating KA2 receptor subunit surface expression.

Materials and methods

Antibodies and DNA constructs. The IL-2 receptor α -chain (Tac) monoclonal antibody 7G7 (ATCC, Manassas, VA), GluR6/7 antisera (Bob Wenthold, NIDCD/NIH, Bethesda, MD), and KA2 antibody (Chemicon) were used for immunofluorescence and immunoblot analyses. All fluorescent secondary antibodies were obtained from Molecular Probes (Eugene, OR). The following cDNA constructs were obtained as gifts: KA2 and GluR6 in mammalian expression vectors (P. Seeburg, Max Planck, Heidelberg, Germany) and Tac (Juan Bonifacio, NICHD/NIH, Bethesda, MD). Both wild-type and mutant KA2 and GluR6 C-termini were amplified by PCR. The amplified tails were subcloned into the cytosolic portion of Tac using *Xba*I and *Eco*RV restriction sites as previously described [16]. Site-directed mutagenesis was performed according to the QuickChange protocol (Stratagene). The sequences of all PCR products and mutants were confirmed by automated sequence analysis.

Immunofluorescence colocalization assay. HeLa cells (ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% L-glutamine, and 0.1% gentamicin, and incubated at 37°C in a 5% CO₂ atmosphere. HeLa cells grown on glass coverslips were transfected with the cDNAs indicated (4 µg of DNA per well, six-well dish) using the calcium phosphate co-precipitation method and analyzed 48 h later. Transfected cells were washed in PBS and fixed in 4% paraformaldehyde in PBS for 15 min at room temperature. The cells were then washed in PBS and permeabilized in 0.25% Triton X-100 in PBS for 5 min. The coverslips were 'blocked' in 10% normal goat serum in PBS for 1 h and incubated with anti-Tac and anti-KDEL antibodies for 1–2 h at room temperature. Following multiple washes with PBS, the cells were incubated with Alexa 568

anti-rabbit secondary antibodies (1:500) and Alexa 488 anti-mouse secondary antibodies (1:500) for 30 min at room temperature, washed, and mounted with ProLong Antifade Kit (Molecular Probes; Eugene, OR). The cells were visualized using a Zeiss Axioplan microscope and pictures were taken with a 63× objective.

Glycosidase treatment and immunoblotting. Glycosidase treatment was performed as previously described [19]. Briefly, transfected HeLa cells plated on 10 cm dishes were collected in lysis buffer (TBS: 2 mM EDTA, 0.1 mM AEBSF 4-(2-aminamide)benzenesulfonyl fluoride, 1 µg/ml leupeptin, and 5 mM iodoacetamide) without detergent, sonicated, and total membranes were collected. The membranes were resuspended in 30 µl of lysis buffer containing 1% SDS and heated at 37°C for 20 min. Five volumes of cold 1% octyl glucoside was then added to each sample and each sample was divided three ways: (i) untreated; (ii) endoglycosidase H (Endo H) (2500 U); or (iii) with *N*-glycosidase F (1250 U). All samples were incubated at 37°C for 4 h and then frozen until further analysis. Samples were thawed, resolved by SDS-PAGE, transferred onto PVDF membranes, and probed with either anti-KA2 (2 µg/ml) or anti-GluR6/7 (1 µg/ml) antibodies. The blots were then incubated with anti-rabbit HRP secondary antibodies (1:5000; Amersham), followed by detection with ECL reagents (Pierce).

Electrophysiology methods. One to three days after plating, HEK293 cells were transfected with plasmids (0.8–1 µg) using Superfect (Qiagen) according to the manufacturer's instructions. To identify transfected cells, 0.2 µg of a plasmid containing CD8 (kindly provided by Prof. Michel Lazdunski, IPMC, Valbonne, France) was also included with the plasmid of interest. To identify CD8-positive cells, immediately prior to recordings the cells were briefly incubated with polystyrene beads coated in anti-CD8 antibody (Dynal). Whole-cell patch-clamp recordings were made two to three days after transfection using electrodes (3–5 MΩ) containing (mM): 117 CsMeSO₄, 8 NaCl, 10 HEPES, 0.2 EGTA, 4 Mg-ATP, and 0.3 Na-GTP, pH 7.4, 290 mOsm. The extracellular solution contained (mM): 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 25 HEPES, and 30 D-glucose, pH 7.3. Recordings were made in voltage-clamp mode using an Axopatch 200-B with a holding potential of –70 mV and filtered at 5 kHz. Series resistance and input resistance were monitored using a –2 mV step applied before each agonist application. Data were digitized at 10 kHz, displayed, and analyzed on-line using the LTP program (www.ltp-program.com). Agonist was rapidly applied by continuous local perfusion using an assembly of four glass pipettes (each of external diameter of 1.5 mm) attached to a piezoelectric element driven by the acquisition software. One barrel contained extracellular solution and a neighboring barrel contained extracellular solution plus 300 µM kainate. The control barrel was placed in front of the recorded cell with the perfusion to all barrels turned off. Perfusion was commenced and then the agonist was applied by rapidly moving the agonist barrel in front of the cell for 200 ms and then returning to the control barrel. For each cell, agonist was applied at least 10 times. For experiments with cells expressing KA2 or KA2 mutants, interleaved experiments with cells expressing either GluR6 or GluR6/KA2 were performed. For each construct at least five cells were tested from at least three different cultures.

Results and discussion

ER-retention of KA2

The kainate receptor subunit KA2 does not form functional homomeric channels [20]. However, it is unknown if this is due to an inability of KA2 to traffic to the plasma membrane or if KA2 homomeric channels are accurately targeted to the plasma membrane, but do not gate current. To address this question, we compared

GluR6 and KA2 expression in HeLa cells. Kainate receptors are known to be glycosylated on asparagine residues [21,22]. We therefore used endoglycosidase H (Endo H) to determine whether or not these receptors contained mature, complex carbohydrates when expressed in HeLa cells. Endo H distinguishes between

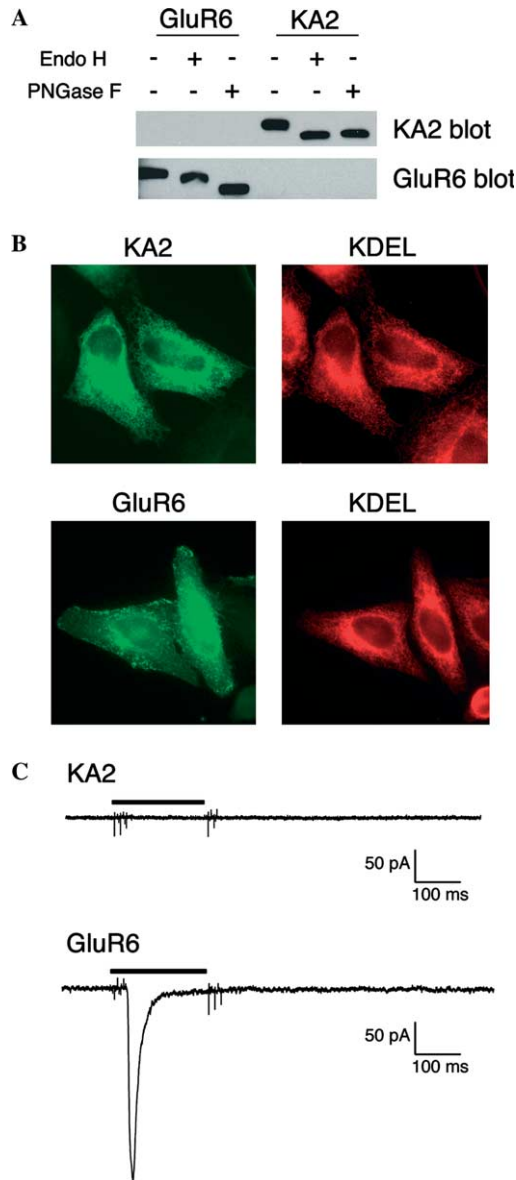


Fig. 1. KA2 is ER-retained when expressed in heterologous cells. (A and B) HeLa cells were transfected with cDNAs encoding GluR6 or KA2. (A) Membrane proteins were collected and incubated alone, with Endo H, or with *N*-glycosidase F, and resolved by SDS-PAGE. Immunoblots were probed with anti-KA2 antibodies or GluR6 antibodies as indicated. (B) The cells were fixed, permeabilized, and incubated with KDEL mouse monoclonal antibodies and either KA2 or GluR6 rabbit polyclonal antibodies as indicated. Immunoreactivity was visualized using anti-mouse Alexa 568 (red) and anti-rabbit Alexa 488 (green). Pictures were taken with a 63 $\times$ objective. (C) Averaged whole-cell current in response to kainate (300 μ M; black bar) from HEK-293 cells expressing either KA2 (upper) or GluR6 (lower; for this and following figures, each trace is an average of 5–8 consecutive applications of kainate).

glycoproteins that are retained in the ER/*cis*-Golgi (Endo H-sensitive) and those that have acquired complex carbohydrate modifications and have at least traversed the medial Golgi (Endo H-resistant). We found that KA2 is completely Endo H sensitive when expressed alone, whereas GluR6 is partially Endo H sensitive (Fig. 1A). This is evidence to suggest that KA2, unlike GluR6, does not traffic beyond the ER when expressed in HeLa cells.

To evaluate the localization of KA2 more precisely, we used immunofluorescence microscopy. For these studies we visualized the ER using an anti-KDEL antibody, a reagent that recognizes retention motifs present in luminal ER-resident proteins [13]. We found strong colocalization of KA2 with KDEL-containing proteins (Fig. 1B), confirming ER-retention of KA2. In contrast, GluR6 displayed a diffuse predominantly plasma membrane expression that did not colocalize with KDEL-containing proteins. To analyze the functional properties of homomeric KA2 and GluR6, we made whole-cell recordings from HEK-293 cells expressing either GluR6 or KA2 (Fig. 1C). GluR6-expressing cells reliably produced robust inward currents in response to kainate application. However, in cells expressing KA2, kainate application never induced a current, consistent with the lack of plasma membrane expression.

GluR6 masks ER-retention of KA2

GluR6 and KA2 coassemble [22] and form functional heteromeric channels [20,23]. To determine if KA2 can

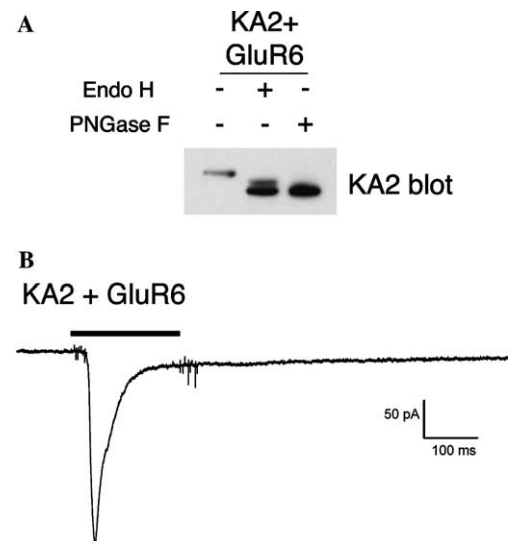


Fig. 2. KA2 traffics to the plasma membrane when co-expressed with GluR6. (A and B) HeLa cells were transfected with cDNAs encoding GluR6 and KA2. (A) Membrane proteins were collected and incubated alone, with Endo H, or with *N*-glycosidase F, and resolved by SDS-PAGE. Immunoblots were probed with anti-KA2 antibodies. (B) Averaged whole-cell current in response to kainate (300 μ M; black bar) from a HEK-293 cell expressing KA2 and GluR6.

traffic to the plasma membrane when assembled with GluR6, we co-expressed the two subunits in HeLa cells and analyzed Endo H sensitivity. We observed a pronounced size shift in Endo H treated KA2 when co-expressed with GluR6 (Fig. 2A), revealing that KA2 was no longer completely Endo H sensitive. These data demonstrate that co-expression with GluR6 obscures an ER-retention motif in KA2 and allows protein transport through the Golgi apparatus. This is consistent with GluR6 enabling the release of KA2 from the ER and subsequent trafficking to the plasma membrane. In HEK-293 cells co-expressing KA2 and GluR6 kainate application produced robust inward currents (Fig. 2B). These data demonstrate that co-expression with GluR6 allows surface expression of functional kainate receptors containing the KA2 subunit.

The KA2 C-terminus contains an ER-retention motif

Ionotropic glutamate receptors have cytosolic C-termini that contain regulatory motifs and sites of protein-protein interactions. For example, the NR1 and NR2 NMDA receptor subunits contain ER-retention motifs within their cytosolic C-termini [16–18]. To determine if ER-retention of KA2 is also dependent on its cytosolic C-terminus, we constructed chimeric proteins of the plasma membrane protein Tac with either the C-terminus of KA2 (TacKA2) or the C-terminus of GluR6 (TacGluR6). Tac-tail chimeras have been used extensively to identify intracellular sorting motifs in proteins

[24–26]. The chimeric proteins were expressed in HeLa cells and their subcellular localization was determined. We found that TacGluR6 was expressed on the plasma membrane (Fig. 3), demonstrating that there is no dominant intracellular sorting signal in the C-terminus of GluR6. In contrast, TacKA2 was highly colocalized with the ER-resident KDEL-containing proteins. Thus, both full-length KA2 (Fig. 1) and TacKA2 (Fig. 3) are ER-retained, revealing that the C-terminus of KA2 contains a dominant ER-retention motif.

NR1 and KA2 have distinct ER-retention motifs

NMDA receptors require both an NR1 and an NR2 subunit to traffic to the plasma membrane [27]. Similarly,

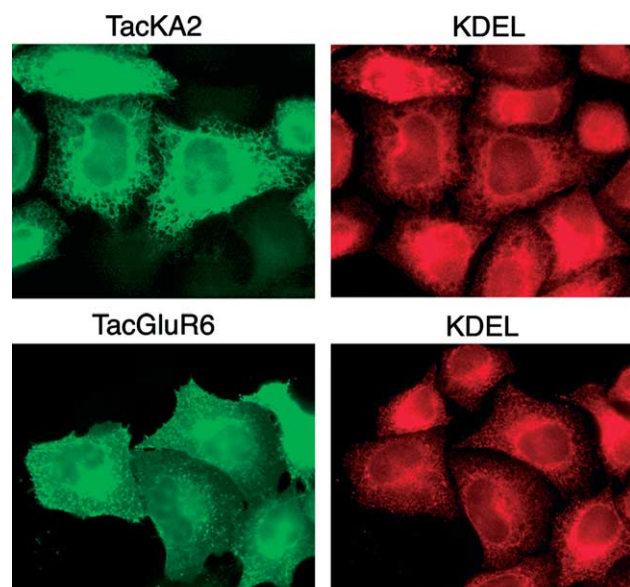


Fig. 3. The C-terminus of KA2 contains a dominant ER-retention motif. HeLa cells were transfected with cDNAs encoding TacGluR6 or TacKA2. The cells were fixed, permeabilized, and incubated with KDEL monoclonal antibodies and either KA2 or GluR6 rabbit polyclonal antibodies. Immunoreactivity was visualized using anti-mouse Alexa 568 (red) and anti-rabbit Alexa 488 (green). Pictures were taken with a 63× objective.

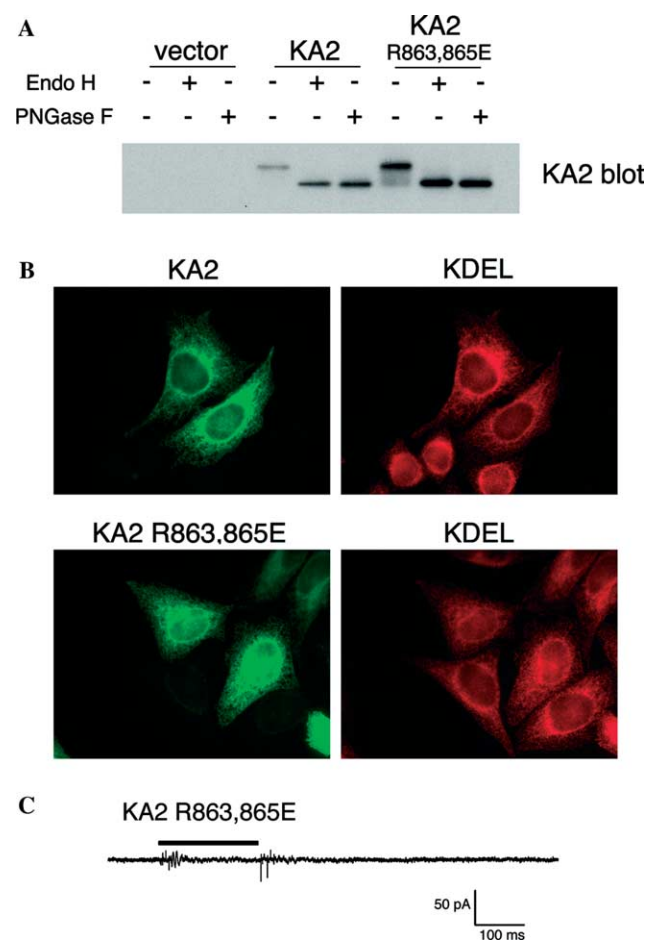


Fig. 4. Disruption of the positively charged motif in KA2 does not alter ER-retention of KA2. (A and B) HeLa cells were transfected with empty vector, wild-type KA2, or KA2 R863,865E. (A) Membrane proteins were collected and incubated alone, with Endo H, or with *N*-glycosidase F, and resolved by SDS-PAGE. Immunoblots were probed with anti-KA2 antibodies. (B) The cells were fixed, permeabilized, and incubated with KDEL mouse monoclonal antibodies and KA2 rabbit polyclonal antibodies as indicated. Immunoreactivity was visualized using anti-mouse Alexa 568 (red) and anti-rabbit Alexa 488 (green). Pictures were taken with a 63× objective. (C) Averaged whole-cell current in response to kainate (300 μM; black bar) from a HEK-293 cell expressing KA2 R863,865E.

we have found that the kainate receptor subunit KA2 does not traffic to the plasma membrane unless co-expressed with GluR6. A positively charged ER-retention motif (RRR) has been identified in the C-terminus of the NR1 subunit [16–18] and mutation of this tri-basic motif with an internal glutamic acid residue (RER) disrupts ER-retention of this subunit [16]. Interestingly, the C-terminus of KA2 contains a similar motif (RRRRR) that is therefore a strong candidate for regulating the ER-retention of this protein. A thorough analysis of both flanking and internal amino acid substitutions affecting the RXR motif indicates internal negatively charged amino acids strongly disrupt ER retention [28]. Therefore, to evaluate the importance of this stretch of amino acids, we mutated two of the internal arginines to glutamic acids (KA2 R863,865E) resulting in five amino acids with alternating charges (RERER). We next examined the subcellular localization of KA2 R863,865E using several different assays. We found that like wild-type KA2, KA2 R863,865E is completely Endo H sensitive (Fig. 4A), consistent with retention of this mutant in the ER. To identify the intracellular localization of this KA2 mutant, we expressed the protein in HeLa cells and found that it colocalizes almost perfectly with the ER marker KDEL (Fig. 4B). In recordings from HEK-293 cells expressing KA2 R863,865E no current was evoked by kainate (300 μ M; Fig. 4C), similar to wild-type KA2. Together these data demonstrate that the mutation of the RRRRR motif into a motif with alternating charges (RERER) does not release KA2 from the ER in heterologous cells, indicating that ER-retention of KA2 is differentially regulated as compared to a protein with the single canonical RXR motif such as NR1.

There is ample evidence to suggest that the expression of glutamate receptors on the post-synaptic membrane is tightly regulated by local plasma membrane mechanisms such as endocytosis and exocytosis [29,30]. In addition, regulation of glutamate receptor trafficking through the secretory pathway is also proving to be a critical checkpoint for surface expression. For example, the binding of certain isoforms of the protein Homer results in the ER-retention of the metabotropic glutamate receptors mGluR1a and mGluR5 [19,31,32]. Furthermore, quality control of the assembly of heteromeric ionotropic glutamate receptor complexes is also regulated by ER-retention of misfolded or inappropriately assembled receptors [16–18]. Our current findings demonstrate that the kainate receptors have similar regulatory mechanisms, including the ER-retention of subunits that have not been assembled into functional heteromeric complexes. In this case the ER-retention motif can be masked by the formation of an oligomeric complex of KA2 with GluR6. Like NMDA receptors, KA2 retention in the ER is an excellent example of

control in the secretory pathway that ensures appropriate synaptic expression of functional receptor complexes assembled in the precise stoichiometry.

Acknowledgments

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