Analysis of Arg834Gln and Val902Glu Type 2A von Willebrand Disease Mutations. Studies With Recombinant von Willebrand Factor and Correlation With Patient Characteristics

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Type 2A von Willebrand disease (vWD), the most common qualitative form of vWD, is characterized by a relative decrease in circulating intermediate and high molecular weight (HMW) multimers. We studied the biosynthesis of recombinant von Willebrand factor (vWF) containing each of two type 2A vWD mutations previously reported by us, Arg834Gln and Val902Glu. The structure of recombinant Arg834Gln vWF within transfected COS-7 cells and the secretion of HMW multimers were similar to wild type vWF. The normal transport and secretion of Arg834Gln vWF, categorizes it as a group II type 2A mutation. In contrast, the Val902Glu mutation resulted in intracellular proteolysis of vWF with the generation of a 176-kD fragment and retention of vWF between the endoplasmic reticulum and the Golgi complex. Moreover, the 176-kD fragment was also increased in plasma from patients with the Val902Glu mutation. Significantly impaired secretion and intracellular proteolysis of Val902Glu vWF categorizes a new sub-group of type 2A mutations. The intracellular proteolysis of vWF Val902Glu explains the lack of response to 1-deamino 8-D-arginine vasopressin (DDAVP) in patients who carry the mutation. © 1996 by The American Society of Hematology.

VON WILLEBRAND FACTOR (vWF) is a multimeric plasma glycoprotein that plays several important roles in hemostasis.1 vWF binds platelets to the subendothelium at sites of vascular injury. In addition, vWF carries and stabilizes factor VIII in the circulation. The vWF gene, located on chromosome 12, spans 178 kb and is interrupted by 51 introns.2,3 vWF is synthesized from an 8.7-kb mRNA and is expressed in endothelial cells and megakaryocytes. Synthesis of vWF is a complex, multistep process that results in assembly of multimers of up to 100 subunits.4 Studies in endothelial cells indicate that dimerization by disulfide bonding at carboxy-terminal domains occurs in the endoplasmic reticulum, whereas further multimerization takes place in Golgi or post-Golgi compartments through disulfide linkages at amino-terminal domains. In the blood vessel, vWF is constitutively secreted by endothelial cells. vWF is also stored within intracellular granules in both endothelial cells (Weibel-Palade bodies) and platelets (α-granules). These specialized granules release vWF in response to a variety of stimuli including vascular damage. The vWF stored within these granules contains larger multimers than those that are constitutively secreted by endothelial cells. These high molecular weight (HMW) multimers appear to be most effective in platelet binding, 5,6 therefore, rapid release of stored vWF into the circulation may be particularly useful at times of vessel injury.

Defects in vWF result in von Willebrand disease (vWD), the most common inherited bleeding disorder in humans. vWD is a phenotypically heterogeneous disorder with over 20 distinct subtypes identified. However, these subtypes can be divided into two major categories, depending on whether there are quantitative (type 1 and type 3) or qualitative (type 2) defects in vWF.7 Type 2A vWD, the most common qualitative form of disease, is characterized by a decrease in circulating intermediate and HMW vWF multimers and is usually inherited in an autosomal dominant pattern. An additional frequent characteristic of this subtype is the relative increase in plasma of a 176-kD vWF carboxy-terminal proteolytic fragment,8,9 that is a result of calpain-like cleavage of the peptide bond between amino acid residues Tyr-842 and Met-843 in the vWF subunit.10,11 To date, 17 missense mutations responsible for dominant type 2A vWD have been identified.12,13 All but two of these mutations are clustered within the A2 homologous repeat of the vWF subunit. The proteolytic cleavage site that generates a 176-kD proteolytic fragment associated with type 2A vWD plasma is located in close proximity to the mutations.10 Expression of recombinant vWF containing type 2A mutations has elucidated two distinct pathogenic mechanisms for type 2A vWD: impaired intracellular transport of HMW multimers (group I mutations) and increased sensitivity to extracellular proteolysis resulting in degradation to lower molecular weight vWF forms in plasma (group II mutations).11 In this report we characterize the biosynthesis of recombinant vWF containing each of two type 2A vWD mutations previously reported by us, R834Q and V902E.13 Based on the expression data, the R834Q mutation can be categorized as a group I type 2A mutation that is characterized by extracellular proteolysis of vWF, whereas the V902E mutation represents a new group of type 2A mutations that is characterized by a striking degree of intracellular proteolysis. This particular mutation, V902E, appears to be associated with a poor clinical response to DDAVP.

MATERIALS AND METHODS

T4 DNA polymerase, T4 polynucleotide kinase, and protein A agarose beads (CL-4B) were purchased from Pharmacia Inc (Piscataway, NJ). Sequencing kit including Sequenase was purchased from United States Biochemical (Cleveland, OH). T4 DNA ligase, calf intestine alkaline phosphatase, N-acetyl-leucyl-leucyl-norleucinal, and restriction endonucleases were from Boehringer Mannheim (In-
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dianapolis, IN). Taq DNA polymerase (Amplitaq) and polymerase chain reaction (PCR) buffer were from Perkin Elmer-Cetus (Norwalk, CT). Endoglycosidase H (EndoH) was purchased from New England Biolabs (Beverly, MA). Deoxyadenosine 5'-[α-32P]-thiotriphosphate (labeled ATP) and γ[32P]ATP were from New England Nuclear (Boston, MA). DEAE-dextran, aprotinin, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St Louis, MO). Agarose and Seaplaque agarose were purchased from FMC BioProducts (Rockland, ME), Geneclean glass beads from FMC BioProducts (Rockland, ME), Geneclean glass beads from Dynabeads, M-280, Dynal AS (Oslo, Norway). Complete Dulbecco’s modified Eagle Medium (DMEM), DMEM lacking methionine and cysteine, and fetal calf serum (FCS) were from Gibco-BRL (Gaithersburg, MD). Anti-vWF polyclonal antibodies 082 and 101 were from Dako (Carpinteria, CA). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer model 380A or 380B (Applied Biosystems, Foster City, CA).

Methods

Plasmid constructs. Plasmid pSVHvWF1 contains a full-length cDNA for human vWF cloned into the expression vector pSV7D, as previously described.15 Two mutamids derived from pSVHvWF1 were constructed by introducing the following mutations: a G to A transition at S040 (numbering starting from the major transcription cap site which is 250 nucleotides before the first nucleotide of the initiation codon)1 resulting in Arg834 to Gln (pSVR834Q). A T to A transversion at S244 resulting in Val902 to Glu (pSVV902E) (amino acid numbering starting from the initiator methionine).1

The previously described nucleotide changes were introduced into pSVHvWF1 by sequential PCR.10 To construct pSVR384Q, two sets of external normal primers 1 and 2 (primer 1, sense: 5’-GAAGACTGTCCAGTGTG-3’, first nucleotide position-3833; primer 2, antisense: biotin-5’-GAGCGGCCCTATATTG-3’, first nucleotide position-5432) and internal mutant primers 3 and 4 (primer 3, antisense: 5’-TCCTGTCACTCGCTGCT-3’, first nucleotide position-5046; primer 4, sense: 5’-GGGTGACCAGGA-GCAGG-3’, first nucleotide position-5035) were used. First, two DNA fragments were amplified using primers 1 and 3 or primers 2 and 3, respectively. Then, the generated DNA fragments were mixed and further amplified using external primers 1 and 2. Similarly, the pSVV902E was constructed using a set of external primers 1 and 2 and internal mutant primers 3 and 4 (primer 3, antisense: 5’-AGCTCCAGTCGAGAGCG-3’, first nucleotide position-5250; primer 4, sense: 5’-TGACCTGGAACGTCAGA-3’, first nucleotide position-5239). The DNA fragments generated from amplification with primers 1 and 5, or 2 and 6, were mixed and further amplified by the external primers 1 and 2. The resulting linear PCR fragments were digested with Nae I and Xmn I enzyme to release 0.75% agarose gels, excised, and purified on Geneclean glass beads. The DNA sequence of each of the mutated plasmids was verified by direct sequencing, using biotinylated primers and an automated sequencer.

Plasmid pSVV902E was constructed using a set of external primers 1 and 2. The resulting linear PCR fragments were digested with Nae I and Xmn I enzyme to release 0.75% agarose gels, excised, and purified on Geneclean glass beads. The DNA sequence of each of the mutated plasmids was verified by direct sequencing, using biotinylated primers and an automated sequencer.

Expression of wild-type (WT) (pSVHwWF1), pSVR384Q, and pSVV902E. COS-7 cells were transfected with 20 μg of wild-type or mutant plasmids by the DEAE-dextran method as previously described.17 Twenty-four hours after transfection, cells were washed twice in phosphate-buffered saline (PBS) and cultured in 8 mL of serum-free DMEM. Medium was collected after 48 hours and EDTA, PMSF, leupeptin, aprotinin, and pepstatin were added to final concentration of 1 mM, 1 mM, 10 μg/mL, 10 μg/mL, 10 μg/mL, and 10 μg/mL, respectively. To examine steady-state levels of vWF, cells were first detached from plates with 0.1% (w/v) trypsin followed by washes with PBS and lysed in 2 mL of Nonident P 40 (NP-40) lysis buffer (150 mMol/L NaCl, 50 mMol/L Tris-HCl pH 7.5, 0.5% sodium dodecyl sulfate [SDS], 1% NP-40, 5 mMol/L EDTA, 2 mMol/L iodoacetate acid, 2 mMol/L N-ethylmaleimide and 2 mMol/L PMSF) per T-75 culture flask. For pulse-chase experiments, cells were lysed directly from plates in 2 mL of NP-40 lysis buffer per 100-mm plate. vWF derived from cell lysates (2 mL per flask) and conditioned media (8 mL per flask) was quantified by a sandwich enzyme-linked immunosorbent assay (ELISA)17 and used for steady-state analysis. Conditioned media was further concentrated by ultrafiltration (Centriprep-30 and Centricron-100, Amicon Inc, Beverly, MA) and used for SDS-agarose multimer gel electrophoresis.

Protein characterization. SDS-Agarose multimer gel electrophoresis was performed as previously described.17 ELISA. vWF was quantitated by a sandwich ELISA using 1:1,000 rabbit anti-human vWF (Dakopatts A082) as the coating antibody and 1:5,000 peroxidase-conjugated anti-vWF antibody (Dakopatts P226) as the detecting antibody. ELISAs were developed with 0-phenylenediamine as the colorimetric substrate and quantitated at A492 on an ELISA reader (Molecular Devices, Menlo Park, CA).17

Pulse-chase labeling. Forty-eight hours posttransfection, COS-7 cells were washed twice with PBS and placed in DMEM lacking methionine and cysteine for 1 hour. Cells were then pulsed for 15 to 30 minutes with 250 μCi each of [35S] methionine and [35S] cysteine per 100 mm plate in 2 mL of DMEM minus methionine and cysteine with 0.5% bovine serum albumin (BSA). Labelling medium was then removed and 4 mL of complete DMEM containing 0.5% BSA and 1% insulin/transferrin/selenium supplement was added, and cell medium and cell lysates were collected at various chase periods.14

Immunoprecipitation. vWF was immunoprecipitated (IP) as described previously14 from 1 mL of cell lysate or 1 mL of cell medium derived from pulse-chase experiments in immunoprecipitation buffer (10 mMol/L Tris-HCl, pH 7.5, 1 mMol/L EDTA, 150 mMol/L NaCl, 0.5% Triton X-100 [Sigma], 0.5% sodium deoxycholate) with 500 ng of rabbit anti-human vWF IgG fraction, followed by incubation with 50 μL of protein A-agarose beads. In some of the IP experiments with the V902E mutant, the above antibody was replaced by the 33E12 anti-vWF monoclonal antibody (MoAb) (a gift from Claudez Maurier, Lille, France). This antibody recognizes the SP II C-terminal fragment of vWF.11

Endoglycosidase H digestion. COS-7 cells were transfected with vWF plasmids and labeled with [35S] methionine and [35S] cysteine, as described under “Pulse-chase labeling.” After 48 hours of labeling, vWF was immunoprecipitated from cell lysates and medium, as previously described. The vWF was eluted from protein A-agarose beads with 25 μL of denaturing buffer (0.5% SDS, 1% β-mercaptoethanol). Samples of immunoprecipitated cell lysate or medium (12.5 μL) were heated to 100°C for 10 minutes. Endo-H digestion was performed with 1,000 U of Endo-H in 50 mMol/L sodium citrate, pH 5.5, overnight at 37°C. Undigested samples were identical treated without the addition of Endo-H. The samples were analysed on 5% reducing SDS-polyacrylamide gels followed by autoradiography.

Quantitative analysis of vWF from cell lysates and cell medium. COS-7 cells transfected with WT and each of the mutant constructs were pulsed and cell lysates and cell medium were collected at various chase periods, immunoprecipitated, electrophoresed, and autoradiographed as previously described. Quantitation of [35S]-labeled bands of the autoradiographs were performed at 633 nm using a densitometer (model 300A; Molecular Dynamics, Sunnyvale, CA).
Within each pulse-chase experiment, the number of counts was expressed in arbitrary units after subtraction of background of 750.

**Protein gel electrophoresis.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli using 5% gels. Samples were placed in a 100°C water bath for 3 minutes immediately before loading on gels. Polyacrylamide gels containing 35S-labelled samples were fixed and treated with Enhance (DuPont, Wilmington, DE). Gels were then dried and exposed to Kodak XAR-5 film (Eastman Kodak, Rochester, NY).

**Immunopurification of plasma vWF.** Using the method described by Zimmerman et al, vWF was immunopurified from plasma with anti vWF MoAb 5.5.72, coupled to cyanogen bromide-activated Sepharose CL4B at a density of 4 mg of IgG per mL of beads. vWF was reduced with 65 mmol/L dithiothreitol for 15 minutes at 60°C in the presence of 2% SDS, electrophoresed in SDS-5% polyacrylamide gels, followed by immunoblotting with anti vWF antibody and 125I-rabbit antimouse antibody as described previously. The relative proportions of the vWF fragments of 189, 176, and 140 kD and of the intact subunit of 225 kD were determined by cutting out radioactive bands from the nitrocellulose blots and quantitating radioactivity in a γ-scintillation counter.

**RESULTS**

**Steady-State Analysis of Recombinant vWF Containing Type 2A vWD Mutations R834Q and V902E**

The previously identified type 2A vWD mutations G5040A (R834Q) and T5244A (V902E) were introduced into full-length vWF cDNA in the expression vector pSV7D. To examine the synthesis of vWF containing these mutations, COS-7 cells were transfected with the pSVHvWF1, pSVR834Q, and pSVV902E plasmids, as described in Materials and Methods, and vWF in cell media and in cell lysates from transfected cells was quantitated by ELISA. Comparisons of the vWF level in conditioned media between WT and each mutant showed that the levels of vWF R834Q secretion was 80% of WT, whereas the level of vWF V902E was only 5% of WT secretion (Fig 1). Intracellular levels of vWF R834Q were only slightly decreased compared to that of WT, however, the intracellular level of vWF V902E were greater than that of vWF R834Q or WT.

**Multimer Analysis of Secreted Recombinant vWF**

To assess the effect of the mutations on multimer structure, the multimer pattern of the recombinant vWF secreted from COS-7 cells transfected with WT and plasmids containing R834Q or V902E mutations. Different volumes of vWF from conditioned media were adjusted so that each lane contains 30 ng vWF, and analyzed on nonreducing 1.5% SDS-agarose gel: 5 μL of WT vWF, 10 μL of R834Q vWF, and 100 μL of V902E. The V902E lane is derived from a separate gel that was processed at identical conditions to the gel that contained the NP, WT, and R834Q lanes.

**Pulse-Chase Analysis of Recombinant Normal and Mutant vWF Proteins**

To further examine the cellular mechanism for these different levels of secreted vWF, pulse-labeled vWF in cell media, and lysates was immunoprecipitated for analysis by electrophoresis and autoradiography. vWF WT was detected in the medium after 2 hours (Fig 3A). The faint bands of either WT or R834Q vWF become clear after prolonged exposure (Fig 3A and B). The mutant vWF R834Q was secreted at a slightly decreased rate compared to vWF WT, as implied from the quantitative analysis by densitometry shown in Table 1. Unlike WT vWF or vWF R834Q, vWF...
V902E was detected in the cell medium only after prolonged exposure (Fig 3A). The vWF R834Q mutant contained a single pro-vWF species in cell lysates (Fig 3C) that appeared identical to WT on reducing gels and was Endo-H-sensitive (Fig 4). In contrast, an additional band of approximately 176 kD was observed in cell lysates derived from transfection with pSVV902E (Fig 3C). The 176-kD band was entirely sensitive to digestion with endoglycosidase H (Fig 4) indicating that it was retained in a compartment before the medial Golgi. This fragment was precipitable with MoAb 33E12 (data not shown) indicating that it consists of the C-terminus of vWF. A similar proteolytic fragment of 176 kD was shown to be caused by a calpain-like activity in plasma. However, the addition of a cell-permeable inhibitor of calpain (50 μg/mL N-acetyl-leucyl-leucyl-norleucinal) to cell medium at all pulse-chase steps did not reduce the generation of the 176 kD proteolytic fragment within the COS cells (data not shown). In this experiment a 5-minute pulse did not detect a full-length vWF before the generation of 176-kD fragment. The residual N-terminal fragment has never been detected in conditioned media, probably indicating that it has undergone intracellular degradation.

Quantitative analysis of vWF from cell lysates derived from pulse-chase (Table 1) was performed by densitometry as described in Materials and Methods. Intracellular V902E mutant showed more vWF in the 176-kD band than in the pro-vWF band. Both bands accumulated and then decreased in parallel, indicating that the mutant proteins are degraded in an intracellular compartment (Fig 3C, Table 1). To visualize the low levels of secreted V902E mutant vWF, the film containing 35S-labeled bands representing cell media, underwent prolonged exposure (14 days) (Fig 3B) and then densitometry of the overexposed film was performed, as described in Materials and Methods (Table 1). As shown in Fig 3B, COS cells transfected with either normal or mutant plasmids secreted both pro and mature vWF. A discernible 176-kD band was detected in media from pSVV902E transfection at 48 hours. This band was clearly visible after 14 days exposure (Fig 3B). As shown in Table 1, at 24 hours, COS cells transfected with WT plasmid secreted 1.3-fold more vWF.

| Table 1. Quantitative Analysis of vWF in Cell Lysates and Conditioned Media |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                             | Chase Period (h)            |                             |                             |
|                             | 0.5 | 2   | 6   | 24 |
| Cell lysate                 | WT  | R834Q| V902E| 176 kD |
| WT                          | 14,570 | 12,881 | 6,186 | 10,427 |
| R834Q                       | 11,187 | 12,198 | 3,977 | 9,082 |
| V902E                       | 10,585 | 10,895 | 3,867 | 7,924 |
| Media                       | WT  | R834Q| V902E| 176 kD |
| Pro-vWF                     | 53  | 61  | 6  | 53  |
| vWF                         | 600 | 568 | 147 | 2,435 |
| Pro-vWF                     | 2,000 | 1,302 | 324 | 4,307 |
| vWF                         | 2,435 | 3,294 | 565 | 5,553 |
| Pro-vWF                     | 836 | 719 | 180 | 395 |
| vWF                         | 840 | 840 | 395 | 395 |

Quantitative analysis of intracellular WT and mutant vWF. vWF from cell lysates and conditioned media, derived from pulse-chase experiments was immunoprecipitated, electrophoresed, and autoradiographed. Radiolabeled vWF bands were quantitated by densitometry, as described in Materials and Methods. Analysis of representative experiment is shown. Within each pulse-chase experiment the number of count is expressed in arbitrary units.
than cells transfected with R834Q plasmid and 18-fold more vWF than cells transfected with V902E plasmid. All vWF species in the cell medium including the proteolytic 176-kD band were endo-H-resistant (Fig 4) indicating that they represented secreted forms rather than the release of intracellular vWF.

**Subunit Composition of Plasma vWF**

The two previously reported patients with the V902E mutation are from unrelated families. Patient P.G. (number 2 in Fig 5) is a 44-year-old woman with positive history for bleeding episodes mainly at mucosal sites. Patient B.A. (number 3 in Fig 5) is a 44-year-old man with positive history for mucosal and after minor surgery bleeding. Both patients’ laboratory data are consistent with a diagnosis of type 2A vWD. DDAVP infusion in both patients did not normalize either the bleeding time or ristocetin cofactor.

Examination of the subunit composition of patients’ plasma vWF showed a decrease in the proportion of the intact 225-kD subunit accompanied by a significant increase in the proportion of the 176-kD and 140-kD fragments, whereas the 189-kD fragment was not significantly different from normal (Fig 5). Native fragments of the 189 and 176 kD are derived from carboxy-terminus, whereas the 140-kD fragment is from amino-terminus as previously reported by Berkowitz et al.

**DISCUSSION**

To date, a total of 17 dominant type 2A vWD mutations have been reported. Of these mutations, 9 have been characterized by transfection studies. Group II type 2A mutations are characterized by normal secretion of vWF multimers that are abnormally sensitive to proteolytic degradation in the circulation, resulting in type 2A multimeric...
pattern and decreased vWF function. The R834Q mutation in the present report occurs in the same codon as the R834W mutation reported previously to be classified as a group II mutation. However, group I or II type 2A vWD mutations cannot be distinguished on the basis of the location or type of amino acid substitution. For example, the group II mutation G742E reported previously occurred in the same codon as the group I defect G742R and immediately adjacent to a second group I mutation, S743L.14

In this report we present transfection analysis of recombinant vWF containing R834Q or V902E mutations. The multimeric structure of R834Q vWF analyzed by SDS-agarose gel electrophoresis was similar to that of the wild type vWF, showing full range of multimers. Also, the pattern of the cellular processing of R834Q mutant was identical to that of the WT vWF. Thus, R834Q mutation can be classified as a group II type 2A mutation; this group also includes R834W, G742E, I865T, and E875K.14,22,23

In contrast to vWF R834Q, the V902E mutant exhibited a profound quantitative and qualitative defect in transport and secretion. Only low molecular weight forms were secreted and the intracellular subunits were proteolytically degraded to a major 176-kD fragment. Based on the endoglycosidase H studies, most of this 176-kD fragment was retained intracellularly before the medial Golgi and only a small amount was secreted by the cells. These properties also differ from those of group I type 2A mutations, including G742R, S743L, L777P, and V844D.14,22,23 Group I mutations are associated with abnormal vWF transport and secretion, but not with intracellular proteolysis. Therefore, we suggest that the V902E mutation represents a distinct category of vWD type 2A mutations that cause extensive intracellular proteolysis of the mutant subunits.

Normal plasma vWF contains small amounts of 176-kD subunit fragments that begin at residue Met843, suggesting that vWF catabolism involves proteolysis at specific sites10,11; the responsible protease has not been identified. In many patients with vWD type 2A, the mutation causes increased proteolytic cleavage and large amounts of 176-kD fragment are found in plasma vWF.10 For patients with group II mutations, this proteolysis occurs extracellularly in the circulation. This is consistent with the finding that the loss of HMW multimers can be reduced in some patients by collection of blood in the presence of protease inhibitors.24,25 In the present report, vWF V902E was shown to be degraded intracellularly to generate a similar 176-kD fragment, suggesting that a similar protease could be responsible, or that the Tyr842-Met843 peptide bond is especially susceptible to attack by a variety of intracellular and extracellular proteases. Alternatively, an intracellular protease may cleave a distinct peptide bond of the mutant vWF subunit. Because the V902E mutation results in T to A transversion, an alternative splicing generated by the mutation was considered. However, this was excluded by looking either for a splice donor consensus sequence at the mutation site or branch point 18-40 nucleotides upstream of the mutation.

In this report we have also showed the correlation between the transfection data and patient characteristics. Thus, the 176-kD fragment that was generated during intracellular processing of V902E vWF was increased in plasma from 2 patients with the V902E mutation.

The precise pathogenetic mechanism of vWD type 2A mutations may have important implications for therapy. It was reported previously that some type 2A vWD patients respond to DDAVP26 whereas others documented no clinical responses.27 The three patients reported to have the V902E mutation13 did not respond to DDAVP (Prof P.M. Mannucci, Dr G. Castaman, personal communication, May 1995). It is possible that patients with impaired secretion of HMW vWF will not benefit from treatment with DDAVP and, therefore, alternative therapy may be considered for them.

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