Isolation of a Highly Specific Ligand for the α5β1 Integrin from a Phage Display Library
Erkki Koivunen, Bingcheng Wang, and Erkki Ruoslahti
Cancer Research Center, La Jolla Cancer Research Foundation, La Jolla, California 92037

Abstract. Our previous studies showed that the α5β1 integrin selects cysteine pair-containing RGD peptides from a phage display library based on a random hexapeptide. We have therefore searched for more selective peptides for this integrin using a larger phage display library, where heptapeptides are flanked by cysteine residues, thus making the inserts potentially cyclic. Most of the phage sequences that bound to α5β1 (69 of 125) contained the RGD motif. Some of the heptapeptides contained an NGR motif. As the NGR sequence occurs in the cell-binding region of the fibronectin molecule, this sequence could contribute to the specific recognition of fibronectin by α5β1. Selection for high affinity peptides for α5β1 surprisingly yielded a sequence RRETAWA that does not bear obvious resemblance to known integrin ligand sequences. The synthetic cyclic peptide GACRRETAWACG (*CRRETAWAC*) was a potent inhibitor of α5β1-mediated cell attachment to fibronectin. This peptide is nearly specific for the α5β1 integrin, because much higher concentrations were needed to inhibit the α5β1 integrin, and there was no effect on α5β2- and α5β3-mediated cell attachment to vitronectin. The peptide also did not bind to the α1β1 integrin. *CRRETAWAC* appears to interact with the same or an overlapping binding site in α5β1 as RGD, because cell attachment to *CRRETAWAC* coated on plastic was divalent cation dependent and could be blocked by an RGD-containing peptide. These results reveal a novel binding specificity in the α5β1 integrin.

Fibronectin is the only known protein ligand for the α5β1 integrin (Pytela et al., 1985). This binding is mediated by the RGD sequence in the 10th type III repeat of fibronectin (Pierschbacher and Ruoslahti, 1984). Studies with mutated forms of fibronectin have suggested that regions in the 8th and 9th type III repeats are also needed for the full adhesive activity of fibronectin (Obara et al., 1988; Aota et al., 1991; Nagai et al., 1991). These synergistically acting regions may contribute to the specific recognition of fibronectin by α5β1.

The α5β1 integrin is important in promoting the assembly of fibronectin matrix and initiating cell attachment to fibronectin (Akiyama et al., 1989; Zhang et al., 1993). Moreover, α5β1 also appears to play crucial roles in cell migration (Bauer et al., 1992, 1993; Zhang et al., 1993) as well as tumor invasion and metastasis (Gehlsen et al., 1988; Humphries et al., 1986; Saiki et al., 1989; Seftor et al., 1993). Probing of α5β1 function with RGD-containing peptides has suffered from the drawback that these peptides also bind to several other integrins such as the α5β2 and α5β3 vitronectin receptors and the α5β3 fibrinogen receptor of platelets (Ruoslahti and Pierschbacher, 1987).

Peptide libraries (Smith and Scott, 1993) offer a way of searching for peptides with improved binding affinities and selectivities for integrins. In previous experiments, we searched for peptides binding to α5β1 from a hexapeptide library expressed on the surface of filamentous phage (Koivunen et al., 1993). Among the several phage sequences that bound to α5β1 was the cyclic peptide GA*CRGDC*LGA. This peptide, while not specific for α5β1, has a 10-fold higher affinity for α5β1 than the standard linear RGD peptides such as GRGDSP.

The hexapeptide library, even though capable of presenting peptides containing disulfide bonds, seemed to have only a limited repertoire of such peptides. We, therefore, constructed a heptapeptide library in which a random heptapeptide insert is flanked by a cysteine residue on each side. A disulfide bond formed by the cysteines cyclized the random sequences, and as a result the library expresses conformationally constrained peptides capable of high-affinity interactions with receptors. Potent ligands for the α5β1 integrin have been isolated from such a library based on a cyclic hexapeptide (O'Neil et al., 1992). We describe here novel α5β1-binding sequences selected from the cyclic heptapeptide library.

Materials and Methods

Materials

The fUSE 5 virions (Scott and Smith, 1990) and Escherichia coli strains...
K91 kan (Smith and Scott, 1993), K802, and MCI061 were obtained from Dr. G. Smith (University of Missouri, Columbia, MO). Human plasma fibronectin was from the Finnish Red Cross and was iodinated as described (Morla and Ruoslahti, 1992). A 110-kD fragment of fibronectin was prepared as previously described (Pierschbacher et al., 1983). Vitronectin was purified from human plasma as described (Taitgoho et al., 1988). Glutaraldehyde was from Sigma Immunochemicals (St. Louis, MO). Peptides were synthesized on a Applied Biosystems model 430A synthesizer (Foster City, CA) by standard Merrifield solid phase synthesis protocols and t-butoxycarbonyl chemistry. Peptides were cyclized by oxidizing with 0.01 M K2Fe(CN)6 at pH 8.4 and purified by reverse-phase HPLC. To renature cyclic peptides, the disulfide bond was reduced and alkylated as described (Koivunen et al., 1993).

The α5β3 integrin was isolated from human placental extracts made in TBS buffer containing 0.1 M sucrose, 1 mM MnCl2, 1 mM CaCl2, and protease inhibitors (Pytela et al., 1987). The α5β3 integrin was isolated from the same extract using immunofluorescence chromatography with polyclonal antibodies against the cytoplasmic tail of α5 subunit (Giancotti and Ruoslahti, 1990). α5β3 bound to the affinity column was eluted with 0.1 M imidazole containing cytoplasmic tail peptide (Giancotti and Ruoslahti, 1990) in TBS buffer containing 25 mM octyl glucoside. The integrin was purified further using wheat germ agglutinin-Sepharose as described (Pytela et al., 1987). The α5β3 integrin was isolated from outdated platelets (Pytela et al., 1986).

Construction of Heptapeptide Library

A heptapeptide library displaying cyclic sequences was constructed on fuse 5 vector essentially as described (Scott and Smith, 1990). The peptides are expressed at the NH2 terminus of phage surface protein III in this vector. Single stranded DNA was prepared from fuse 5 virions and transfected into K802 cells by electroporation. Double-stranded fuse 5 DNA was extracted from K802 cells with a plasmid preparation kit (Qiagen Inc., Chatsworth, CA) and further purified by CsCl density gradient centrifugation. The insert was prepared by polymerase chain reaction amplification and BglI digestion to synthetic oligonucleotide (Scott and Smith, 1990) that contained a core sequence TGT(3NKN)TGT (N = equal molar mixture of A, C, G, T; K = G or T). The degenerate oligonucleotide was purified from 15% polyacrylamide gel and ligated to fuse 5 vector previously cleaved with SalI. About 24 μg of the ligated vector was transformed into MCI061 cells by electroporation with BglI electroporator and booster according to the instructions of the manufacturer (GIBCO-BRL, Gaithersburg, MD). After culturing the bacteria for 24 h, phage were prepared from 3.2 liter of culture medium by precipitating twice with polyethylene glycol. The procedure yielded a primary library containing 4.5 × 1010 clones. Sequencing of DNA extracted from a small aliquot of the library indicated the expected distribution of bases.

Selection of α5β3-binding Phage

An aliquot of the library containing 2.5 × 1011 transducing units (TU) was screened with α5β3 coated on microtiter wells as described (Koivunen et al., 1993). In the first and second panning the coating concentration of the integrin was 5 μg/well. To increase the stringency of the panning, the wells were coated with decreasing concentrations of the integrin. Phage were selected for further amplification from the well with the lowest integrin concentration that showed phage binding over background. In the third and fourth panning, the concentrations were 10 and 1 μg/well, respectively. To rescue the bound phage, the wells were eluted with a 0.1 M glycine buffer, pH 2.2, containing 1 mg/ml bovine serum albumin and 0.1 mg/ml of phenol red. Phage were sequenced from randomly selected clones as described (Koivunen et al., 1993).

Phage Attachment Assay

Binding of individual cloned phage to integrins was studied essentially as described (Koivunen et al., 1993) except that the entire assay was done in microtiter wells. Phage were incubated for 1 h at room temperature in microtiter wells coated with α5β3 or α5β1. The wells were washed five times and 20 μl of concentrated K91 kan bacteria (Smith and Scott, 1993), diluted 1:5 in Terrific broth media (Sambrook et al., 1989), was added into the wells and incubated for 10 min at 37°C. After adding 100 μl of Terrific broth containing 0.2 μg/ml of tetracycline and 100 μg/ml of kanamycin, the incubation was continued for an additional 30 min at 37°C. Another 100 μl of Terrific broth containing 50 μg/ml of tetracycline and 100 μg/ml of kanamycin was added and the microtiter plate was incubated at room temperature overnight with shaking. The absorbance indicative of bacterial growth was read at 600 nm with an ELISA reader.

Fibronectin-binding Assay

The binding of [125I]-labeled fibronectin to α5β1 coated on plastic was performed as described (Koivunen et al., 1993).

Cell Attachment Assay

Cell lines expressing different integrins were used in cell attachment assays to examine peptide inhibition of integrin function. A human melanoma cell line C8161 (Seftor et al., 1993), a fibroblast cell line WI-38 (Vogel et al., 1990), and an osteosarcoma cell line MG-63 (Pytela et al., 1985) attach to fibronectin through the α5β3 integrin. In some assays fixed concentrations that resulted in 60% maximum attachment for the test cells were used. Peptide was coated on plastic by incubating for 2 h at 37°C in phosphate buffered saline containing 0.25% glutaraldehyde to crosslink the peptide. Any free binding sites on plastic were blocked with bovine serum albumin. Approximately 1 × 105 cells per well were allowed to attach for 1 h in the presence or absence of competing peptides and the bound cells were determined by staining with 0.1% amido black (Ruoslahti et al., 1982).

Results

Phage Selected by α5β1 Integrin Binding Display RGD and NGR Motifs

A majority (69 of 125) of the phage bound to α5β3, from the cyclic peptidemide library contained the RGD sequence in the insert (Table I). The RGD sequences were found in three positions of the heptapeptide, in the middle, next to the NH2-terminal cysteine, or one residue removed from it. Selection of high affinity clones by decreasing the coating concentration of α5β3-favored clones that contained a glycine residue after RGD. Moreover, the glycine was followed by an aromatic residue, tryptophan or phenylalanine, in those sequences. No enrichment of any particular amino acids was noted at the positions preceding the RGD.

Another α5β3-binding motif detected was the RGD analogue NGR, and its variation, NGH (Table II). NGR was found in eight peptides. One NGR-containing clone displayed a sequence VNGRMW which is quite similar to the sequence A LNGREE present in the 9th type III repeat of human fibronectin (Kornblihtt et al., 1985). The sequence ASVNGHT in which the arginine of NGR was replaced by histidine was detected in four clones.

Other Non-RGD-type Sequences

The other α5β3-binding non-RGD sequences derived from the cyclic peptidemide library were more heterogeneous, but could be classified into five groups (Table II). Represen-

1. Abbreviations used in this paper: *CRGDC*, cyclic GACRGDCGLA peptide; *CRRATEAWAC*, cyclic GACRRETAWACG Peptide; IC50, half-maximal inhibitory concentration; TU, transducing unit.
Table I. RGD-containing Sequences in Phage Isolated by αβ3 Binding for a Cyclic (CX3C) Phage Display Library

| Integrin coating concentration for plastic |
|-------------------------------------------|
| 50,000 ng/ml (2nd panning) | 1,000 ng/ml (3rd panning) | 100 ng/ml (3rd panning) | 10 ng/ml (4th panning) | 10 ng/ml (5th panning) | 1 ng/ml (5th panning) |
| LSRGDTP | I PRGDGW (5) | I PRGDGW (3) | ELRGDGW (2) | I PRGDGW (2) | I PRGDGW |
| DRRGDGF | RSRGDFP | YRRGDGH | EYRGDFG | VARGDGW | MTRGDGF |
| FTRGDAP | VARGDGW | ELRGDGW | VARGDGW | QTRGDGW | LFRGDGW |
| TSRGDMP | TRGDGDF (3) | VARGDGW | | |
| QLRGDDG | FRGDGDFK (2) | TRGDGDF (3) | | |
| EDRGDDW | FRRGDGFPE | EGRDLRM (2) | | |
| TLRGDDN | RRGDGDFWE | TRGDQW | | |
| HLRGDDG | RGDWPNY | QRGDGW | | |
| MLRGDFS | LRGDDFL | | | |
| MPRGDGF | YRGDHL | | | |
| SRQGDGF (2) | LRGDAF | | | |
| FRRGDHR | TRGDQWP | | | |
| GRGDSVP | GRGDRCQ | | | |
| SRQGDGF | KRGDFGW | | | |
| GRQDNLP | RGDDFSMM | | | |
| RGDLRFN | | | | |

Selection and sequencing of phage bound to the αβ3 integrin were performed as described in Materials and Methods. The number of clones encoding the same peptide is shown in parentheses.

Table II. Non-RGD Sequences in CX3C Phage Bound to αβ3 and Their Comparison to Fibronectin Sequences

| Type III repeat | Phage sequences | TGLDSPT | ANSFTVH | VPHSRSN | ALNGREESP | ? |
|----------------|----------------|---------|---------|---------|-----------|---|
| 9th            | STSDVGG (3)    | LNTNLGF (2) | EIVKSSS | WANGRSR (3) | RRETAWA (15) |
|                | PELFVES        | GPCGSKS | FVNGRSF (2) | FANGRH | RGAPRAW |
|                | FAGSLLV        | NLTLSV | VLNGME | YVNGVR | |
|                | RFGSHVP        | TLVPSR | WLNGRN | |
|                | SRPSTFL        | | MANGRL | |
|                | SVANSVV        | | | |
|                | ASFFAVQ        | | | |
|                | HVLASAF        | | | |
|                | VFSIAH         | | | |
|                | LVASMTP        | | | |
|                | IGTFHHN        | | | |
|                | AFYQGLP        | | | |
|                | QNAFGYS        | | | |
|                | LGEFAFA        | | | |

Phage bound to the αβ3 integrin were isolated as described in Materials and Methods. The amino acid residues that are common in each group are highlighted by bold. The number of sequences encoding the same peptide is indicated in parentheses. The fibronectin sequences are shown in the order as they occur in type III repeats, from NH2-terminus to COOH terminus. The sequences at analogous positions of the 9th and 10th repeats are aligned. The residues underlined show homology to phage sequences.
phage, indicating that the sequences have a low affinity towards $\alpha_5\beta_1$.

**A Novel High-affinity Peptide Ligand for $\alpha_5\beta_1$**

Surprisingly, the most common sequence detected after the high affinity selection was not an RGD peptide but a peptide with a sequence RRETAWA. The RRETAWA sequence was progressively enriched when the binding conditions for phage were made more stringent by decreasing the amount of integrin available on plastic. The sequence was detected in 4 of 38, 1 of 7, and 10 of 25 total sequences obtained in the third, fourth, and fifth pannings, respectively. The same nucleotide insert encoded the peptide, suggesting a clonal origin.

We synthesized the cyclic peptide GACRRETAWACGA (*CRRETAWAC*) and compared its activity to GA*CRGDC-LGA (*CRGDC*) that had thus far been the most avid peptide binder of $\alpha_5\beta_1$ (Koivunen et al., 1993). *CRRETA-
WAC* inhibited the binding of RRETAWA-containing phage to α5β1, 10-fold better than *CRGDC* (Fig. 1). A control peptide GRGESP had no effect (not shown). *CRRETAWAC* also inhibited the binding of *CRGDC*-containing phage to α5β1, but in this assay the *CRGDC* peptide was a better inhibitor than *CRRETAWAC* (Fig. 2). In contrast, the binding of *CRGDC*-containing phage to the α5β1 integrin could not be inhibited by *CRRETAWAC* (Fig. 3). Consistent with this result, the phage displaying RRETAWA bound only weakly to microtiter wells coated with α5β1, whereas it bound avidly to α5β1 under the same conditions. RRETAWA-phage also did not bind to wells coated with the RGD-directed integrin α5β1, whereas it bound avidly to α5β1 under the same conditions.

The *CRRETAWAC* peptide inhibited the binding of fibronectin to α5β1 (Fig. 4). Half maximal inhibition (IC50) for both the *CRRETAWAC* and *CRGDC* peptide was seen at 8 × 10^-9 M. We also synthesized and tested one NGR-containing peptide A*CVLNGRMEC*G. This sequence was selected because of its similarity to the fibronectin sequence. The NGR-containing peptide with an IC50 of 2 × 10^-7 M was clearly less active than *CRRETAWAC* or *CRGDC* (Fig. 4). However, this cyclic NGR peptide was a much more potent binder of α5β1 than the linear peptide NGRAHA (Koivunen et al., 1993). An unrelated cyclic peptide, GA*CSPYFLRLC*GA, used as a control had no significant effect.

The *CRRETAWAC* Peptide Inhibits Cell Attachment to Fibronectin

The high potency and specificity of *CRRETAWAC* for α5β1, inferred from the integrin-binding assays was confirmed by results from cell attachment assays. *CRRETAWAC* inhibited α5β1-mediated cell attachment to fibronectin at an IC50 of 3 × 10^-3 M, showing similar or slightly higher potency than the *CRGDC* peptide. A control pep-

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tide, GRGESP, had no effect. Fig. 5 A shows the results for a CHO cell line (B2/α27) that expresses the human α5 subunit from transfected cDNA (Bauer et al., 1992). Similar results were obtained with other α5β1-expressing human cell lines studied, C8161, MG-63, and WI-38. The attachment of A375-M cells was only partially inhibited by *CRRETAWAC*, possibly, because this cell line expresses other fibronectin-binding integrins, including α5β1 (Mould et al., 1990). The *CRRETAWAC* peptide also blocked cell attachment to fibronectin, but high concentrations were needed and its activity was more than 100-fold lower than that of *CRGDC* (Fig. 5 B). *CRRETAWAC* showed no inhibition of α5β1- or α5β3-mediated cell attachment to vitronectin, whereas these interactions were readily inhibited by the *CRGDC* peptide (Fig. 5, C and D).

Cell lines expressing the α5β1 integrin attached onto plastic coated with the *CRRETAWAC* peptide (Fig. 6). The α5β1-expressing cells also bound, but to a lesser extent (result not shown). A peptide-coating concentration of 1 mg/ml was needed to produce significant attachment, confirming the weaker interaction of *CRRETAWAC* with α5β1 than with α5β3, indicated by the cell attachment inhibition results presented above. In contrast, cell lines that do not express these integrins (CHO-B2/C1, HT 29) or express nonhuman α5β1 (CHO C11) did not attach to immobilized *CRRETAWAC* (not shown). The cell attachment to *CRRETAWAC* was inhibited by the soluble *CRRETAWAC* and *CRGDC* peptides, and also by EDTA.

**Discussion**

Our screening of a phage display library that was designed to express a random heptapeptide sequence flanked on each side by cysteine residues has resulted in the isolation of a number of new α5β1-binding peptides. These include the heterogeneous group of non-RGD-containing sequences that may be homologous to sequences present in the cell-binding region of fibronectin. Our results also define the first specific peptide ligand for α5β1.

The results presented here indicate that the peptides selected from the cyclic heptapeptide library for binding to α5β1 are more variable than those selected from a linear hexapeptide library. With only a few exceptions, the peptides derived from the linear hexapeptide library we used earlier were RGD peptides independent of whether the phage were eluted by RGD-containing peptide, EDTA, or a low pH buffer (Koivunen et al., 1993). In the present study, we consistently used a low pH buffer as an elution method to not omit any tight binding phase. That many α5β1-binding non-RGD sequences were found in the present study is apparently due to the longer insert in the present library and, more importantly, the cyclic structure of the peptides displayed by it.

Conformationally constrained peptides are known to have an improved affinity for integrins (Pierschbacher and Ruoslahti, 1987; Samanen et al., 1991; Cardarelli et al., 1992; Gurrath et al., 1992; Imura et al., 1992). The importance of the disulfide bond in the sequences we isolated is indicated by the fact that the activity of *CRRETAWAC* peptide was greatly diminished after reduction of the disulfide bond and alkylation of the cysteines. The NGR sequence also had a higher activity when presented in a cyclic peptide. Peptides containing a cysteine pair can also appear in high affinity screens of the linear peptide library (Koivunen et al., 1993). These results and those of O'Neil et al. (1992) suggest that peptides containing two cysteines, when fused to the NH2-terminus of phage protein III are capable of forming the expected disulfide bridge and that the presence of such a bond can greatly enhance the integrin-binding activity of the peptide.

O'Neil et al. (1992) used a CX6C library to isolate cyclic hexapeptides to the α5β1 platelet integrin and found sequences that contained either the RGD or KGD motif. The number of sequences described is small, but there are some notable differences as compared to the RGD sequences we selected for the α5β1 integrin. First, we did not see any KGD sequences among the α5β1-binding clones. That sequence is known to bind only to the α5β1 integrin and is found in some snake venoms (Scarborough et al., 1991). Second, the residue COOH-terminal to RGD often was a large hydrophobic one in the α5β1-binding sequences, whereas we found a strong preference for a glycine residue in the α5β1-binding clones. The glycine was followed by an aromatic tryptophan or phenylalanine. Our recent studies with CX6C and CXsC libraries have similarly indicated that the sequence RGDGW/F is highly enriched in the α5β1-binding phase (our unpublished results). These results show...
that phage display libraries are able to reveal minor differences in the binding specificities of related integrins.

The αβ₂⁄β₃-binding sequences that did not contain the RGD, NGR, or RRETAWA motif could be categorized into four main groups. Each group showed possible homologies to sequences present in the 9th and 10th type III repeats of fibronectin (Table II). The homologous sequences occur at analogous positions in the two type III repeats. Interestingly, all these sequences are likely to reside in exposed loop regions based on the known structure of the 10th type III repeat (Main et al., 1992). Only the serine- and phenylalanine-containing hydrophobic sequence may locate partially in a β strand. The sequences we identified in this manner in the 9th type III repeat could represent the synergistically acting region of fibronectin that has been deduced from site-directed mutagenesis and antibody mapping studies of fibronectin (Obara et al., 1988; Aota et al., 1991; Nagai et al., 1991). These findings suggest a model of fibronectin interaction with αβ₂⁄β₃, that is mediated by cumulative binding of several loop regions of the type III repeats. The interaction at the RGD loop is undoubtedly the strongest, but the NGR and other loops may provide the signal for specific recognition of fibronectin by αβ₂⁄β₃.

The identification of the *CRRETAWAC* peptide as a high affinity ligand for αβ₂⁄β₃, was surprising in that this sequence bears no obvious similarity to fibronectin sequences or other known ligand sequences for αβ₂⁄β₃ or other integrins. Smith and colleagues have also described isolation of peptides from a phage display library that are entirely different from known epitopes for the proteins studied (Smith and Scott, 1993; Smith et al., 1993). Because of its divergent sequence, we were interested to clarify whether *CRRETAWAC* might interact with some other site in αβ₂⁄β₃ than RGD. Four different approaches, phage attachment assay, fibronectin-binding assay, cell attachment to fibronectin, and cell attachment to the *CRRETAWAC* peptide, indicated that *CRRETAWAC* and RGD compete with each other for binding to αβ₂⁄β₃. Moreover, the binding of cells to immobilized *CRRETAWAC* peptide was inhibited by EDTA, indicating that the interaction is divalent cation dependent. However, since the attachment of RSETAωA-bearing phage was inhibited better by the *CRRETAWAC* peptide than by the *CRGDC* peptide and the reverse was true for the *CRGDC*-carrying phage, it is probable that *CRRETAWAC* does not bind at exactly the same site in αβ₂⁄β₃ as RGD, but rather at an overlapping site.

Because we found only one variation of the RRETAWA sequence among the αβ₂⁄β₃-selected clones and because the phage carrying that sequence, RGPRAKW, bound weakly to αβ₂⁄β₃, we do not know which amino acids in *CRRETAWAC* are important for its activity. Since this was the only high-affinity sequence isolated, it is possible that the entire sequence has to remain invariable. The combination of positive and negative charges of *CRRETAWAC* is likely to be important, and the same may be true of the tryptophan residue, because tryptophan was enriched in the RGD peptides selected by high-affinity screening. In any event, the *CRRETAWAC* peptide seems to have an appropriate disulfide-dependent conformation to fit in the binding site of αβ₂⁄β₃.

In conclusion, we have found a novel binding specificity in the αβ₂⁄β₃ integrin. Because of its selectivity and high affinity for αβ₂⁄β₃, the *CRRETAWAC* peptide may be useful to discriminate cell adhesion that is mediated by αβ₂⁄β₃. Immobilized *CRRETAWAC* peptide can selectively promote the attachment of αβ₂⁄β₃-expressing cells. Finally, this work illustrates the potential value of phage display libraries for providing information on the possible contact sites between a ligand and its receptor.

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