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Two Adjacent N-terminal Glutamines of BM-40 (Osteonectin, SPARC) Act as Amine Acceptor Sites in Transglutaminase$_C$-catalyzed Modification*

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The extracellular matrix protein BM-40 (osteonectin, SPARC) has recently been shown to be a major target for transglutaminase-catalyzed cross-linking in differentiating cartilage. In the present study we demonstrate that recombinant human BM-40 can be modified with [^{3}H]putrescine in a 1:1 molar ratio by transglutaminase$_C$ (tissue transglutaminase). Residues Gln^3 and Gln^4 were identified as major amine acceptor sites. This was confirmed with several mutant proteins, including deletions in the N-terminal domain I of BM-40, site-directed mutagenesis of the reactive glutamines, and fusion of the seven-amino acid-long N-terminal sequence (APQQEAL) to an unrelated protein. The results showed that the N-terminal target site is sufficient for modification by transglutaminase but at a low level. For high efficiency amine incorporation an intact domain I is required. The conservation of at least one of the transglutaminase target glutamines in the known vertebrate BM-40 sequences and their absence in an invertebrate homologue point to an important, but yet unknown, role of this modification in vertebrates.

Transglutaminases (EC 2.3.2.13) form a large protein family for which at least six different gene products have been found in higher vertebrates (1). They catalyze a Ca^{2+}-dependent transfer reaction between the γ-carboxamide group of a peptide-bound glutamine residue and various primary amines (2–4). Most commonly, γ-glutamyl-ϵ-lysine cross-links are formed in or between proteins by reaction with the ϵ-amino group of lysine residues. The function of transglutaminase$_C$ (tissue transglutaminase, transglutaminase type II), which is of widespread occurrence in vertebrates (1, 4–6), is not well understood. Its expression often correlates with cellular differentiation (1, 4, 7), and the enzyme may have functions as different as GTP binding in receptor signaling (8), intracellular cross-linking in programmed cell death (9), and extracellular cross-linking in the assembly of matrices (1). It is also involved in wound healing and tissue repair (10). In general the function of transglutaminase$_C$ could be to stabilize protein molecules and protein complexes, thus contributing to the stability of tissues. In skeletal tissues, synthesis of transglutaminase$_C$ is strictly regulated and correlates with chondrocyte differentiation and cartilage mineralization (7, 11). The enzyme is released by hypertrophic chondrocytes (7) and activated by the elevated Ca^{2+} concentration in the extracellular space (11).

BM-40 (osteonectin, SPARC), which is co-expressed with transglutaminase$_C$ in maturing cartilage (7), is a major substrate protein for transglutaminase-catalyzed cross-linking in this tissue (11). BM-40 is widespread in extracellular matrices (12–14). It was initially identified as a major component of bone (15, 16), but is also found in basement membranes (17) and is expressed in platelets (18) and endothelial cells (19). BM-40 has been shown to bind collagen IV in basement membranes (20) and thrombospondin when released from platelets (21). The protein has also been implicated in the mineralization of cartilage and bone because of its affinity for hydroxyapatite (22) due to several calcium-binding sites (23, 24). This 35-kDa extracellular matrix glycoprotein has been predicted to consist of four domains, starting with the acidic N-terminal domain I, followed by a cysteine-rich domain II, an α-helical domain III, and the C-terminal EF hand domain IV (Ref. 23; see Fig. 3*B*). Several deletion and point mutants have recently been used to localize the collagen IV and high affinity calcium binding sites of BM-40 to domains III and IV (25). Tissue-specific post-translational modifications, such as glycosylation (26) or transglutaminase cross-linking (11), may also serve to regulate some of the diverse biological functions proposed for BM-40 (13, 14).

In the present study we have identified Gln^3 and Gln^4 within domain I as the amine acceptor sites in BM-40 for transglutaminase$_C$-catalyzed cross-linking with the primary amine putrescine. The interaction of transglutaminase with BM-40 was analyzed by introducing mutations into domain I. The results indicated that the N-terminal sequence APQQEAL alone is sufficient for modification by transglutaminase but that the complete domain I of the protein is required for efficient modification.

MATERIALS AND METHODS

Protein Reagents—Transglutaminase$_C$ was purified from guinea pig liver (27). The production and characterization of recombinant human BM-40 (28) and its deletion mutant ΔN (amino acids 8–67 deleted, D69-E) (25) have been described previously. The preparation of a protein construct, γ1III3–5 (formerly B2III3–5), containing the N-terminal

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seven amino acids of human BM-40, APQQEAL, connected to EGF[1]-like repeats 3–5 in domain III of the laminin γ1-chain, has been described elsewhere (29). Constructs for two novel BM-40 mutants were prepared by polymerase chain reaction according to the suppliers instructions (Perkin-Elmer) using a human cDNA encoding BM-40 inserted into the Bluescript vector (28, 30). Oligonucleotide primers 5′-GATCGCTAGCAAATCCCTGCCAGAAC and 5′-GATCCTCGAGTTAGATCACAAGATCC were used to amplify a 725-bp fragment encoding amino acids 53–286 of BM-40 in order to produce mutant ΔI which lacks the entire domain I. Primer 5′-GATCGCTAGCAGAAGCCCTGCCTGAT and the second primer (see above) were used to amplify an 869-bp fragment encoding amino acids 3–286 of BM-40 for the production of the mutant QQ3,4-LA containing two amino acid substitutions. Restriction fragments, obtained by digestion with *Nhe*I and *Xho*I, were purified by agarose gel electrophoresis and ligated to the short version of the BM-40 signal peptide Bluescript construct as described previously (29). Correct ligation and in-frame insertion of the fragments was verified by DNA sequencing. The complete inserts were then cut out by digestion with *Xba*I and *Xho*I and ligated into the *Xba*I/*Xho*I site of the eukaryotic expression vector pCis (31).

The transfection of human embryonic kidney cell clones 293 and the selection of stable cell clones by puromycin followed previous protocols (28). The recombinant proteins were purified from serum-free culture medium on DEAE-cellulose and Superose 12 (HR 16/50, Pharmacia LKB Biotechnology Inc., Uppsala, Sweden) columns (25). The purity of the proteins was analyzed by SDS-polyacrylamide gel electrophoresis and by Edman degradation which verified the novel N-terminal sequence APLAEA for mutant QQ3,4-LA.

Radioactive Transglutaminase Assay—Transglutaminase-catalyzed incorporation of [2,3-^{3}H]putrescine (30.2 Ci/mmol, DuPont NEN) into substrate proteins was done for 45 min at 37 °C as described previously (6). The specific incorporation of ^{3}H label was calculated by subtracting the radioactivity in samples which had been incubated in the absence of substrate proteins or proteins known not to be transglutaminase substrates, *i.e.* guinea pig immunoglobulin γ and bovine serum albumin. Saturation experiments to determine the uptake of labeled putrescine per mol of recombinant BM-40 were done as described previously (6). Kinetic constants for putrescine incorporation into BM-40 and mutant γ1III3–5 were determined as described (32). Briefly, rates of incorporation of [^{3}H]putrescine into varying amounts of substrate protein were determined from reactions performed in 0.1 M Tris/HCl, pH 7.4, 30 mM NaCl, 50 mM $CaCl_2$, and 1 mM EDTA, at 25 °C. The conditions were adjusted such that the incorporation was linear over a 10-min period. Putrescine was employed at a set high concentration (1 mM), a level which appeared to be saturating (data not shown). Kinetic constants were derived from fitting initial rates to the following equation.

$$v = k_{cat}[E]_o[S]/(K_m(app) + [S]) \quad \text{(Eq. 1)}$$

Identification of the Amine Acceptor Sites in BM-40 and in Mutant Proteins—Incorporation of [1,4-^{3}H]putrescine (100 μCi/ml; 30.0 Ci/mmol, Amersham Corp., Amersham, United Kingdom) into recombinant BM-40 (10 μM, 1.5 mg) by transglutaminase$_C$ (1 μM, 0.35 mg) was performed in 0.1 M Tris/HCl, pH 8.3, containing 5 mM $CaCl_2$ and 18.9 μM putrescine (Serva Fine Biochemicals, Paramus, NJ), at 37 °C for 45 min. The reaction was stopped by addition of EDTA to a final concentration of 25 mM, and the sample was dialyzed against 6 M guanidine HCl, 0.1 M Tris/HCl, pH 8.0. Radioactively labeled monomeric BM-40 was separated from oligomers and the enzyme by chromatography on a Superose 6 column (FPLC HR 10/30, Pharmacia LKB Biotechnology, Inc.) equilibrated in the same buffer at a flow rate of 0.3 ml/min. The labeled protein was reduced and alkylated as described (33) and digested with 0.26 μM trypsin (enzyme/substrate, 1:100 molar ratio) for 2.5 h at 37 °C in 0.2 M ammonium hydrogen carbonate. The tryptic peptides were separated by reversed phase HPLC on a C_{18} column (Nucleosil, Macherey & Nagel, Düren, Germany, 4 × 250 mm) using 0.1% trifluoroacetic acid (solvent A) and 70% acetonitrile in 0.1% trifluoroacetic acid (solvent B) with a gradient of 0–90% B in 70 min at a flow rate of 1 ml/min. Fractions of 200 μl were collected, and 10% of each fraction was mixed with scintillation fluid (Rotiscint 22, Roth KG, Karlsruhe, Germany) and the radioactivity measured. The tryptic peptides of pool 1 were further cleaved with 1 μg of endoproteinase Asp-N (Boehringer Mannheim, Mannheim, Germany) for 16 h at 23 °C in 0.2 M ammonium hydrogen carbonate. The tryptic peptides in pool 2 were further cleaved with 1 μg of endoproteinase Glu-C (*Staphylococcus aureus* SV8 protease, Boehringer Mannheim) for 5 h at 37 °C in the same buffer. The resulting peptides were separated and the radioactivity determined as above.

The recombinant mutant proteins γ1III3–5 and BM-40 QQ3,4-LA (150 μg; 8.3 and 5.0 nmol, respectively) were labeled with [2,3-^{3}H]putrescine in the transglutaminase-catalyzed reaction at an enzyme/substrate molar ratio of 1:10 and the labeled monomeric proteins purified by molecular sieve chromatography on a Superose 6 column as described above. The labeled proteins were reduced and carboxymethylated under denaturing conditions and cleaved with endoproteinase Glu-C and trypsin, respectively, in 0.2 M ammonium carbonate at 23 °C and an enzyme/substrate ratio of 1:100 for 16 h. The resulting peptides were separated by reversed phase HPLC and the radioactivity was determined as described above.

Radioactive peptides were sequenced using the Applied Biosystems Sequencer model 470A according to the manufacturer's instructions. The small volume of phenylthiohydantion derivative samples left over after on-line injection was collected in the instrument's fraction collector and used for radioactivity determination as described.

Protein Concentration Determination—Protein concentrations were determined using the bicinchoninic acid reagent standard protocol as described by the supplier (Pierce) with bovine serum albumin as a standard or by amino acid analysis after hydrolysis with 6 M HCl (16 h, 110 °C) on a LC3000 analyzer (Biotronik).

RESULTS

Identification of the Substrate Glutamine Residues in BM-40 for Transglutaminase$_C$-catalyzed Modification—We have recently demonstrated that BM-40 is cross-linked by transglutaminase$_C$ in the matrix surrounding hypertrophic chondrocytes during endochondral bone formation and postnatal development of tracheal cartilage in the rat (7, 11). However, BM-40 can be isolated from rat skeletal tissues only in small amounts and under denaturing conditions, whereas the native conformation is required for the protein to act as a transglutaminase substrate (7). We therefore used the human recombinant protein, which has been shown to be fully native (28), to identify the target sites of transglutaminase$_C$ action on this protein. [^{3}H]Putrescine was incorporated into recombinant BM-40 by incubation with guinea pig liver transglutaminase$_C$ in the presence of Ca^{2+} (33). To determine the maximum amount of [^{3}H]putrescine which can be cross-linked to recombinant BM-40, the incorporation of label was driven to saturation by incubation with successively increasing amounts of unlabeled putrescine at a constant concentration of [^{3}H]putrescine (6). The results (Fig. 1) showed that approximately 1 mol of putrescine was incorporated per mol of protein.

For identification of the target glutamine(s), the labeled monomeric protein was separated from oligomers and the enzyme by gel filtration under denaturing conditions, reduced and alkylated, and digested with trypsin. Separation of the tryptic peptides by reversed phase HPLC and determination of [^{3}H]putrescine content in the fractions showed two peaks containing together about 90% of the applied label (Fig. 2*A*). Sequencing of these fractions showed that they contained a mixture of peptides with the N-terminal sequence of the protein as the predominant component in both pools. Pool 1 was further cleaved with endoproteinase Asp-N, and separation of the cleavage products by reversed phase HPLC revealed only one radioactive peak containing 84% of the applied label (Fig. 2*B*). This peak contained a long peptide starting with the N-terminal sequence APQQE and a short peptide comprising positions 212–221 (IFPVHWQFGQ). Determination of radioactivity in the residual volume of injection during sequencing showed ^{3}H label in cycles 3 and 4, but not in cycles 7 and 10. This demonstrated that only Gln^3 and Gln^4 are substrate sites for transglutaminase$_C$. However, as the N-terminal peptide was too long to be sequenced entirely, the second radioactive pool of tryptic peptides, which apparently contained a similar labeled

[1] The abbreviations used are: EGF, epidermal growth factor; HPLC, high performance liquid chromatography; FPLC, fast protein liquid chromatography.

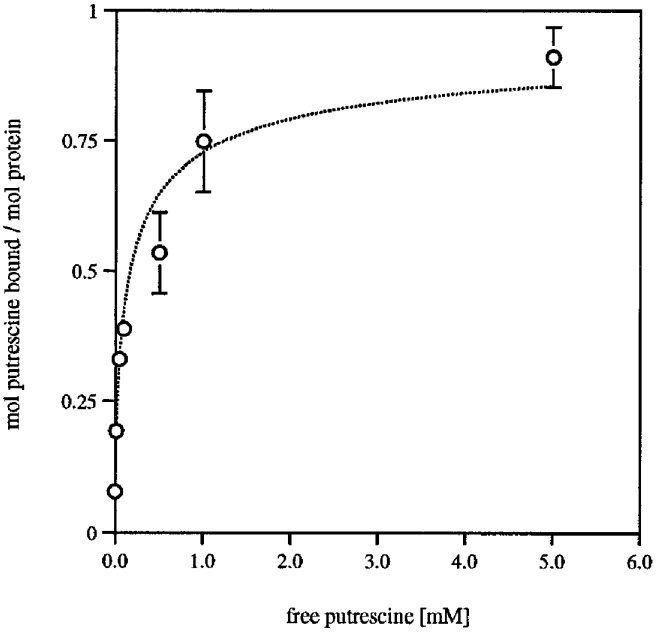

FIG. 1. **Saturation of the amine incorporation site(s) in BM-40.** Increasing amounts of unlabeled putrescine were added to the reaction mixture of recombinant BM-40 and transglutaminase$_C$, whereas the concentration of [^{3}H]putrescine was left constant. The amount of protein-bound radioactivity was measured as described (6). The *circles* represent the mean of four determinations with *bars* indicating the deviation from the mean. Semilogarithmic plots of the data (not shown) demonstrated that saturation was reached.

fragment, was further cleaved with endoproteinase Glu-C. The chromatogram of the reversed phase HPLC separations of these peptides showed one radioactive peak with 75% of the applied radioactivity (Fig. 2*C*). This peak contained only the peptide APQQEALPDETEVVEE, and label was again found only in cycles 3 and 4. The relative recoveries of radioactivity in cycle 3 and 4, corresponding to Gln^3 and Gln^4, varied in the different sequencer runs. Although with the long peptide of pool 1 slightly more radioactivity was found associated with Gln^3 than Gln^4, the reverse was true for the short peptide derived from cleavage of pool 2. The radioactivity released during sequencing in cycle 4, on the other hand, was too high to be explained by carryover of the phenylthiohydantoin-derivative from cycle 3. Therefore, [^{3}H]putrescine was attached to either Gln^3 or Gln^4 of human BM-40 probably to a similar extent. However, because reversed phase HPLC could apparently not separate peptides labeled at either Gln^3 or Gln^4, we were always sequencing mixtures of both.

Influence of Mutations in the N-terminal Domain of BM-40 on Transglutaminase-catalyzed Modification—Mutants were used to study the influence of structural changes in the N-terminal domain of BM-40 on putrescine incorporation (Fig. 3). In mutant QQ3,4-LA, the two target glutamines of intact BM-40 were replaced by Leu and Ala, respectively, whereas the rest of the molecule was unchanged. In mutant ΔI the entire N-terminal domain I of BM-40 was deleted. Mutant ΔN contained a deletion of residues 8–67, thus lacking most of domain I but retaining the first seven amino acids (APQQEAL) of intact BM-40. Finally, the first seven amino acids containing the two target glutamines were fused to an entirely different protein, domain III of the laminin γ1-chain, consisting of three EGF-like repeats in tandem (mutant γ1III3–5). When the mutants and intact BM-40 were labeled with [^{3}H]putrescine under identical conditions, we found (Fig. 4) that mutant ΔI, which lacked the entire domain I, including the essential glutamines, was no longer able to incorporate the label. Mutant ΔN, which

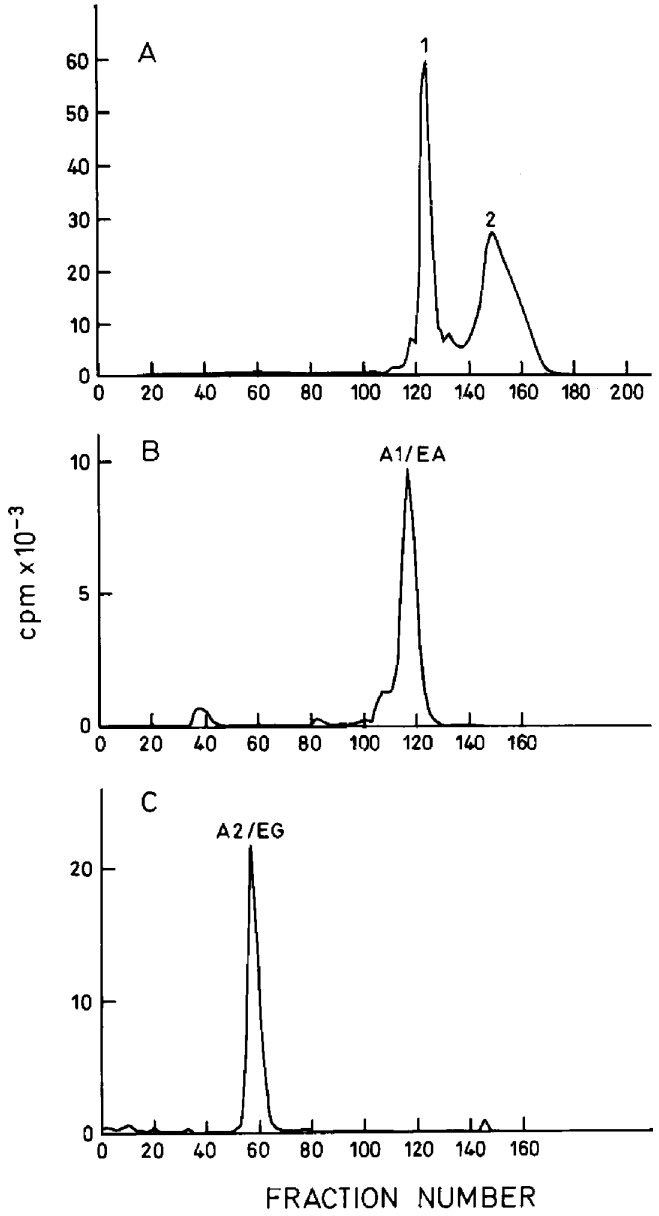

FIG. 2. **Radioactivity profiles of reversed phase separations of peptides derived from cleavage of [^{3}H]putrescine-labeled human recombinant BM-40.** [^{3}H]Putrescine was incorporated into native recombinant BM-40 by transglutaminase$_C$. The labeled protein was purified by molecular sieve chromatography, reduced and alkylated, and digested with trypsin. The separation of the tryptic peptides, resulting in two pools (*1* and *2*) of labeled fragments, is shown in *A*. The separation of products of endoproteinase Asp-N cleavage of pool 1 and endoproteinase Glu-C cleavage of pool 2 are shown in B and C, respectively.

lacked most of domain I except the target sequence was still able to incorporate about 29% as much as intact BM-40. Mutant γ1III3–5, in which only the seven-amino acid-long target sequence of BM-40 was present, still incorporated 12% of the label found in intact BM-40. The substrate specificity factor, k_{cat}/K_m(app), for this reaction was 66 mM^{-1} min^{-1} (k_{cat} = 156 ± 20 min^{-1}, K_m(app) = 2.35 ± 0.26 mM; mean of three experiments with each determination done in triplicate to measure the initial rates), whereas that for the reaction with BM-40 was 146 mM^{-1} min^{-1} (k_{cat} = 3.53 ± 0.24 min^{-1}, K_m(app) = 0.0242 ± 0.0013 mM). Most interestingly, mutant QQ3,4-LA incorporated 23% as much label as intact BM-40, although both target glutamines were lacking.

In order to determine the modified glutamines in these mu-

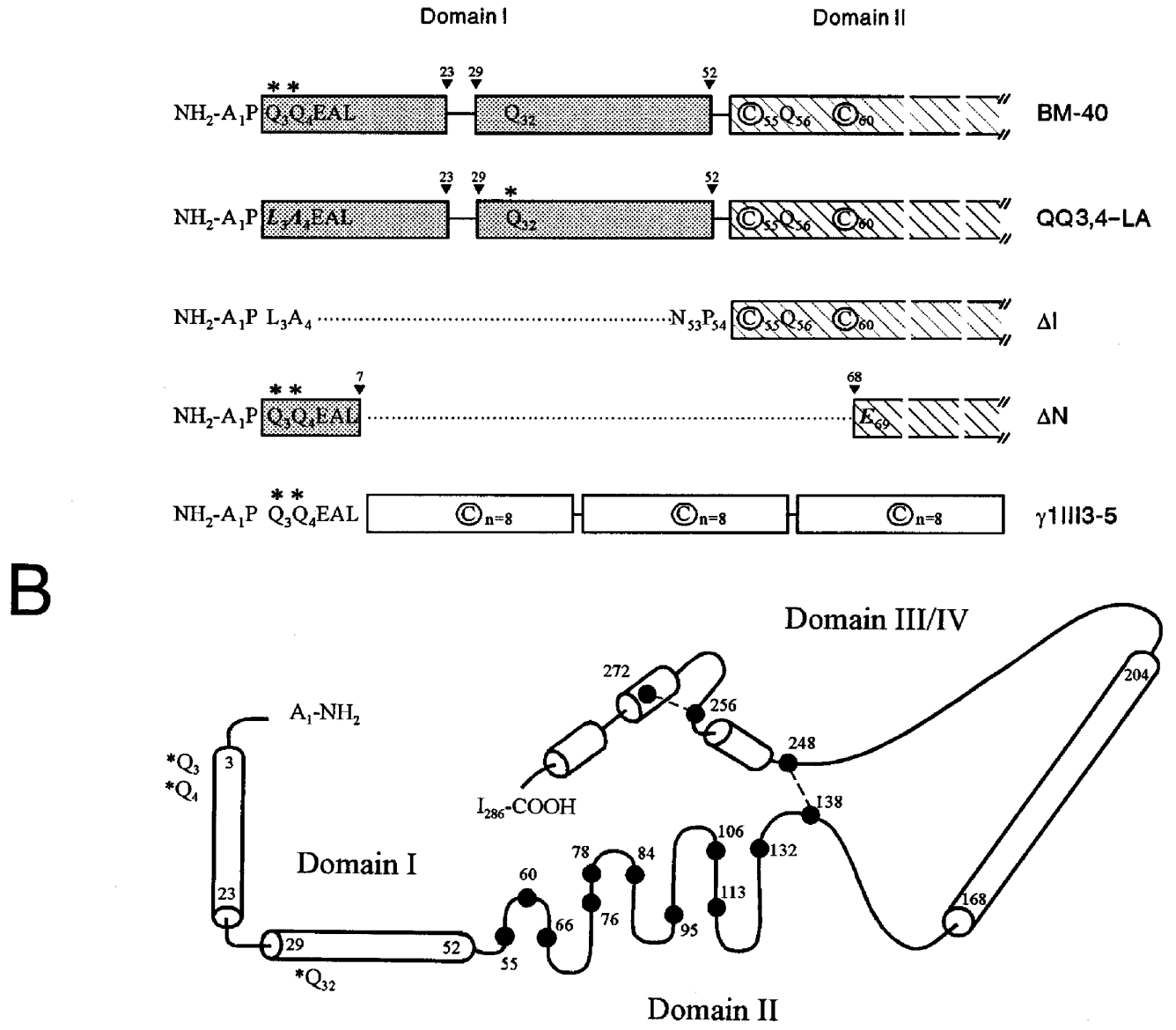

FIG. 3. **Design of mutant proteins and location of transglutaminase amine acceptor sites.** *A*, scheme representing the mutations described in the text. *Gray boxes* indicate domain I of BM-40 with the limits of the proposed α-helical regions (see *B*) marked by *arrowheads*. *Numbers* indicate sequence positions according to Ref. 30. In mutant QQ3,4-LA glutamines Gln^3 and Gln^4 were replaced by Leu and Ala, respectively. Mutant ΔI has a precise deletion of domain I (residues 1–52) but contains the additional N-terminal residues APLA due to the ligation to the signal peptide of human BM-40 using an *Nhe*I restriction site (29). Mutant ΔN lacks residues 8–67 and contains an exchange of Asp^{69} for Glu. γ1III3–5 contains the N-terminal seven amino acids of BM-40 fused to a domain of the γ1 laminin chain containing three EGF-like repeats. *B*, model of BM-40 according to Ref. 23. Cylinders represent predicted α-helical domains, *black dots* indicate cysteines, and *numbers* indicate sequence positions (30). The amine acceptor sites of native recombinant BM-40, Gln^3 and Gln^4, and the alternative site in mutant QQ3,4-LA, Gln^{32}, are marked with an *asterisk*.

tant proteins we isolated and digested the [^{3}H]putrescine-labeled monomeric proteins. Mutant γ1III3–5 yielded several radioactive peaks after cleavage with trypsin and separation of the peptides by reversed phase HPLC. All of these peaks contained peptides of various length starting at the N-terminal sequence APQQ with a total yield of ~55% of the radioactivity applied to the column. Radioactivity was released only in sequencing cycles 3 and 4, indicating that [^{3}H]putrescine was cross-linked to the same glutamine residues as in genuine BM-40. The [^{3}H]putrescine-labeled mutant QQ3,4-LA was cleaved with endoproteinase Glu-C. When the resulting HPLC peaks were screened for radioactivity, only a single peak was found to be radioactive with a yield of about 40%. This peak included only one Gln-containing peptide, VSVGANPVQVE (residues 24–34), in addition to smaller amounts of the peptides RDEDNNLLTE, and DNNLLTE. Only cycle 9 released radioactivity, indicating that Gln^{32} was the residue labeled with [^{3}H]putrescine.

DISCUSSION

Human recombinant BM-40 was used to determine the amine incorporation site for transglutaminase$_C$-catalyzed modification. Proteolytic digestion of [^{3}H]putrescine-labeled BM-40 yielded labeled peptides of variable length that were derived

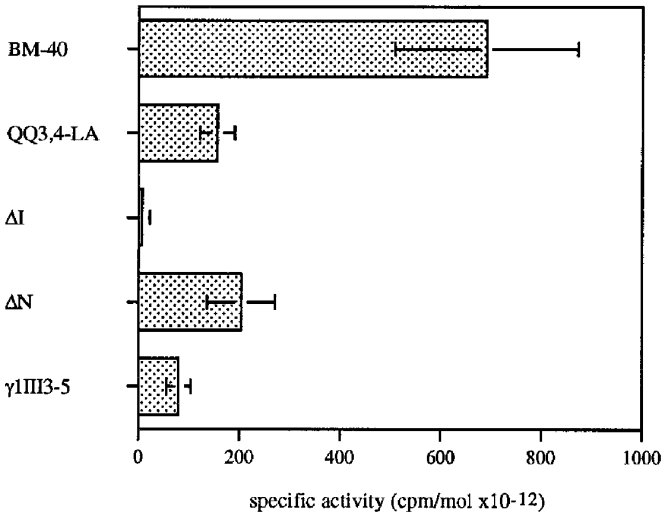

FIG. 4. **Transglutaminase$_C$-catalyzed incorporation of [^{3}H]putrescine into BM-40 and the different mutant proteins.** The incorporation of [^{3}H]putrescine into human recombinant BM-40 and the mutants QQ3,4-LA, ΔI, ΔN, and γ1III3–5 by transglutaminase$_C$ was carried out at 37 °C for 45 min in the presence of 5 mM Ca^{2+} as described previously (6). The results are shown as a mean of 6–18 independent measurements, and the *bars* indicate the standard deviation from the mean.

from the N terminus and had the label attached exclusively to Gln^3 and Gln^4. The transglutaminase did not show a clear preference for either of these glutamine residues but modified only one of them in each BM-40 molecule as indicated by the 1:1 ratio of putrescine incorporation under saturating conditions. Two adjacent glutamine residues were identified as amine acceptor sites in several other proteins, including fibronectin (34) and fibrinogen γ-chain (35) using factor XIIIa, plasminogen activator inhibitor-2 (36), and β A3-crystallin (37) using the guinea pig liver enzyme (transglutaminase$_C$), and involucrin (38) using the keratinocyte enzyme (transglutaminase$_K$). Transglutaminases often show a preference for one of the two residues, although exclusive modification of one of the adjacent residues has not been observed with proteins (discussed in Ref. 33). However, in a study using short peptides patterned on the N-terminal sequence of fibronectin, EAQQIV, only the first Q has been shown to be an amine acceptor in the factor XIIIa or transglutaminase$_C$-catalyzed modification with monodansylcadaverine (39). Both, Gln^3 and Gln^4 are conserved in human, mouse, bovine, and frog BM-40, whereas in rat and chicken only one of the two Gln residues is retained (Table I). The BM-40 homologue from the nematode *Caenorhabditis elegans* lacks these glutamine residues as well as showing no significant overall sequence similarity in the N-terminal domain I (40). The conservation in higher vertebrates of the unique N terminus of BM-40, which contains the transglutaminase cross-linking site and binding sites for Ca^{2+} (24), suggests a specific function of this domain in these species. This could be related to the need for cross-linking of BM-40 in calcifying cartilaginous and osseous tissues, which are absent in almost all invertebrates.

When the N-terminal sequence of BM-40 was grafted onto an entirely different protein, the EGF-like repeats 3–5 of domain III of laminin γ1-chain, this sequence was still modified by transglutaminase$_C$ at the same glutamine sites as genuine BM-40, but the amount of label incorporated was distinctly reduced and the substrate specificity factor (k_{cat}/K_m(app)) was only half of that with recombinant BM-40 as a substrate. A drastic reduction in putrescine incorporation was also found with mutant ΔN, in which most of domain I of BM-40 was missing and the transglutaminase target site was directly connected to domain II. This shows that an N-terminal sequence of BM-40, consisting of seven amino acids, is sufficient to be recognized and modified. However, because the level of modification is much lower than with intact BM-40, the remainder of domain I also seems to play an important role in the interaction between transglutaminase and BM-40. Possibly other amino acids in this domain also contribute to enzyme recognition. The results with mutant QQ3,4-LA indicated that binding of the enzyme to domain I of BM-40 can give rise to the modification of another accessible Gln (Gln^{32}) when the major target residues are lacking. However, the level of modification was significantly lower than for intact BM-40, indicating that the proper distance between the transglutaminase binding site and the target residue is crucial for efficient modification or that the proper conformation of the target sequence depends on the rest of domain I. No sequence similarity is apparent between the N-terminal target sequence and the immediate surroundings of Gln^{32}, the substitute target in mutant QQ3,4-LA. However, the comparison of known transglutaminase target site sequences (33) does not reveal a clear sequence motif which may serve as a signal sequence for modification.

TABLE I
Comparison of N-terminal BM-40 sequences from different species
Dashes have been introduced for better alignment.

Species	Sequence	Ref.
Human	AP QQ EALPDET	30
Rat	AP Q- TEAAEEM	11
Mouse	AP QQ TEVAEEI	19, 30
Bovine	AP QQ EALPDET	16
Chicken	AP Q- EALADET	41
Frog	AP QQ DALPEEE	42
Nematode	DA KK KKIADDE	40

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