Midkine (MK), a retinoic acid-inducible growth/differentiation factor, serves as a substrate for tissue transglutaminase (Kojima, S., Muramatsu, H., Amanuma, H., and Muramatsu, T. 1995. J. Biol. Chem. 270, 9590–9596). Upon incubation with transglutaminase MK forms multimers through cross-linkages. Here, we report the following results. 1) Heparin potentiated the multimer formation by MK. 2) The N- and C-terminal half domains each formed a dimer through the action of transglutaminase. 3) Gln42 or Gln44 in the N-terminal half and Gln95 in the C-terminal half served as amine acceptors in the cross-linking reaction, as judged from the incorporation of putrescine into whole MK or each half domain, and the competitive inhibition of the cross-linking by MK-derived peptides containing Gln residue(s). The strongest inhibition was obtained with Ala4-Asp5. 4) This peptide abolished the biological activity of MK to enhance the plasminogen activator activity in bovine aortic endothelial cells. The inhibition was limited against the MK monomer, and not seen against the MK dimer, separated by gel filtration chromatography. These results suggest that dimer formation through transglutaminase-mediated cross-linking is an important step as to the biological activity of MK.

Tissue type II transglutaminase (R-glutaminylpeptide: amine γ-glutamyltransferase, EC 2.3.2.13) is a member of the transglutaminase family that catalyzes Ca2+-dependent acyl transfer reactions between γ-carboxamide groups of the Gln residues in peptides and either primary amines or ε-amino groups of the Lys residues in peptides, resulting in the formation of new γ-amides of glutamic acid or ε-(γ-glutamyl)lysine bonds and ammonia (1, 2). The molecular structure of tissue transglutaminase has been reported (3–5). Although tissue transglutaminase is widely distributed in the body (6), its physiological function is not well established compared with those of other members of the transglutaminase family, e.g. the formation of cross-linkages between fibrin molecules by plasma Factor XIIIa (1, 2), and the formation of cross-linked envelopes during epidermal cell differentiation by tissue type I transglutaminase (7, 8). Recently, tissue type II transglutaminase was implicated in the association of proteases and protease inhibitors with the cell surface (9, 10), in the activation of several cytokines (11, 12), in signal transduction (13), and in the process of apoptosis (14).

Midkine (MK)1 and pleiotrophin (PTN) constitute a new family of heparin-binding growth/differentiation factors (15, 16). MK has been found as a product of a retinoic acid-responsive gene (17), and exerts a variety of biological activities; it enhances neurite outgrowth and the survival of various embryonic neuron types (18–21), and is mitogenic for certain fibroblastic cell lines (18, 19). In addition, recently, we found that MK enhances the plasminogen activator (PA) activity in bovine aortic endothelial cells (BAECs; Ref. 22). PTN, also called heparin-binding growth-associated molecule (HB-GAM; Refs. 23 and 24), was found as another neurite-promoting factor (25). PTN has been shown to be mitogenic for endothelial cells (26) and to enhance tubule formation in vitro (27). Expression of these factors is strictly controlled during the processes of differentiation and development (28–30). MK is highly expressed in many human cancers (31) and specifically localized in senile plaques of Alzheimer’s disease (32), and the overexpression of PTN in NIH3T3 cells results in transformation of the cells (33), suggesting the involvement of MK and PTN not only in normal development, but also in the pathogeneses of diseases (15, 16). MK is a 13-kDa heparin-binding polypeptide rich in basic amino acids and cysteine (17, 34), and exhibits 46% sequence identity with PTN (15). Both MK and PTN are largely composed of two domains, called the N-half (N1/2; Lys1–Gly59 in human MK) and the C-half (C1/2; Ala60–Asp121 in human MK), each of which contains a couple of intra-disulfide linkages (35, 36). Of these two domains C1/2 is responsible for heparin-binding, neurite outgrowth-promoting, and PA-enhancing activities (37, 38).

During the course of studying the PA-enhancing properties of MK, we discovered that MK serves as a good substrate for tissue transglutaminase (22). BAECs constitutively produce and secrete MK, which forms a transglutaminase-mediated complex in cultures. Prior to this discovery, a 29-kDa MK-related protein, which is now recognized as a dimer of MK, had been detected in a variety of tissues, such as the lymphonode, spleen, testis, small intestine, stomach, lung, kidney, and liver (39). Furthermore, Haynes and colleagues (40, 41) reported similar dimer formation by MK in the developing brain. These
lines of accumulating evidence suggest that the cross-linking of MK by tissue transglutaminase may occur and play an important role in MK biology in vivo.

In the current study, we have investigated the mechanism underlying the transglutaminase-mediated cross-linking reaction and the relevance of dimer formation to MK activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—The procedure for chemical synthesis of human MK and its fragments including N1/2 and C1/2 domains was described in a previous paper (42). Antibodies against the whole MK molecule, N1/2 domain, and C1/2 domain were produced in rabbits by injecting each antigen subcutaneously into animals after homogenization with Freund's complete adjuvant (Sigma). Immunization and bleeding were conducted biweekly. The IgG fraction was isolated from the sera using protein A-Sepharose (Pharmacia Biotech Inc.). The antibodies were shown to be immunospecific to each antigen by Western blotting as reported previously (37) or as shown in Fig. 1. The antibody against whole MK recognized the C-terminal tail (Thr105–Asp121) and thus recognized C1/2, but not N1/2 (37). The anti-N1/2 antibody did not cross-react with C1/2 (Fig. 1, panel A). The antibody to C1/2 recognized C-domain (Cys62–Cys104), which forms the compact structure maintained by two disulfide bonds in C1/2 (Fig. 1, panel B). This antibody showed strong, weak, and no cross-reactivity against C1/2, whole MK, and N1/2, respectively. Guinea pig liver transglutaminase and heparin sodium salt were purchased from Takara Biochemicals (Ohtsu, Japan) and Nacalai Tesque Inc. (Kyoto, Japan), respectively. Purified plasma factor XIII was a generous gift from Dr. Y. Saito (Tokyo Institute of Technology, Yokohama, Japan).

**Putrescine Incorporation Assay**—Intact (whole) MK, N1/2, or C1/2 was incubated with transglutaminase in Hepes-buffered saline (129 mm NaCl, 5 mm KCl, 0.3 mm NaHPO4, 1 mm NaHCO3, 5 mm glucose, and 25 mm Hepes, pH 7.4) containing 10 mm CaCl2, 8 mm dithiothreitol, 0.3% (v/v) glycerol, and 3 mm [14C]putrescine (0.45 μCi, Amersham), in a final volume of 300 μl. The reaction mixture was incubated for the indicated time at 37 °C and then the reaction was stopped by the addition of 600 μl of 16.7% trichloroacetic acid with 100 μl of 2% bovine serum albumin. The precipitate was collected on Whatman GF/C glass filters and washed three times with 2 ml of 10% trichloroacetic acid, and then the radioactivity was measured by liquid scintillation counting.

The amount of putrescine incorporated into each sample was expressed as moles of putrescine incorporated/mol of each sample.

**Western Blotting**—Western blotting was performed as described previously (22) using antibodies to intact MK (final 5 μg/ml), N1/2 (final 100 μg/ml), or C1/2 (final 100 μg/ml), and goat anti-rabbit IgG antibodies conjugated with peroxidase (Jackson ImmunoResearch Laboratories, Ltd., West Grove, PA). The signals were detected with an Amer sham (Buckinghamshire, United Kingdom) ECL system.

**Assaying of Cellular PA Activity**—BAECs were isolated and grown in α-minimal essential medium containing 10% calf serum. The levels of cellular PA activity were measured using the chromogenic substrate, S-2405, as described previously (22), and expressed as urokinase units/mg of protein in each sample.

**Gel Filtration Chromatography Using FPLC**—Gel filtration chromatography was performed using a Superdex 75 HR 10/30 column and a FPLC System (Pharmacia). The column was equilibrated and eluted with Hepes-buffered saline buffer containing 1 m NaCl at the flow rate of 0.5 ml/min. The high salt was included to suppress the nonspecific adherence of MK to the apparatus (43). The absorbance at 280 nm was monitored, and fractions of 0.5 ml each collected.

**RESULTS**

**Enhancement of the Cross-linking of MK by Heparin**—Recently, we found that MK serves as a substrate for tissue transglutaminase (22). Incubation of recombinant murine MK with purified tissue transglutaminase readily yielded SDS- and β-mercaptotoethanol-resistant multimers, as detected by Western blotting (22). A similar result was obtained with synthetic MK (Fig. 2, panel A). In BAECs, tissue transglutaminase functions in part on the cell surface (11), suggesting that cross-linking of MK might also occur in the cell surface milieu. On the other hand, MK is known to have strong heparin-binding activity (15). Therefore, we examined if the heparin-binding activity of MK affected its cross-linking by tissue transglutaminase. As shown in Fig. 2 (panel B), the inclusion of heparin in the reaction mixture significantly enhanced both the rate and the final degree of the formation of MK multimers by transglutaminase.

**Identification of Glnα2, Glnα4, and Glnα5** as Possible Amine Acceptors—The formation of multimers indicates that both murine and human MK contain both Gln and Lys residues that participate in the transglutaminase-mediated bridge formation. Both murine and human MK contain 5 Gln residues/molecule (15, 17). To determine the number of acyl-donating
seen in Fig. 3 (columns 2 and 3), both domains share one Gln site each. This also suggested that N1/2 and C1/2 alone each might be cross-linked by transglutaminase, as both domains contain many acyl-acceptable Lys sites (9 in N1/2 and 14 in C1/2). This was proved by the results of Western analyses performed following the incubation of either N1/2 or C1/2 with transglutaminase (Fig. 4). Although multimers larger than the tetramer were not observed much, a significant amount of the dimer was detected for both N1/2 and C1/2 (lanes 2 and 3). In contrast, comparable amounts of the dimer and tetramer were detected on the cross-linking of whole MK molecules (lane 1). These reactions did not occur in the absence of Ca\(^{2+}\) (data not shown). N1/2 and C1/2 contain two and three Gln residues, respectively (15). Therefore, it is suggested that either Gln\(^{82}\) or Gln\(^{44}\) in N1/2 and one of the three Gln residues (amino acids 82, 93, and 95) in C1/2 serve as acyl-donating sites. Plasma-derived FXIIIa catalyzed the incorporation of \(^{14}\)C putrescine into MK, but did not catalyze the cross-linking of MK molecules, as assessed by Western blotting (data not shown).

In parallel with putrescine incorporation assays, the effect of Gln-containing peptides on the cross-linking of intact MK, N1/2, and C1/2 by transglutaminase was examined. Synthetic human MK, N1/2, or C1/2 (final: 3 \(\mu\)M each) was incubated for 30 min with 1.25 \(\mu\)M tissue transglutaminase and 30 \(\mu\)g/ml heparin in the absence or presence of 300 \(\mu\)M Gln-containing peptides, and then the formation of each dimer was assessed by Western blotting after SDS-PAGE on 14% resolving gels. Panel A, intact MK; panel B, N1/2; panel C, C1/2. Lane 1, no competitor; lane 2, Ala\(^{41}\)–Pro\(^{51}\); lane 3, Thr\(^{78}\)–Gly\(^{83}\); lane 4, Thr\(^{84}\)–Gln\(^{93}\); lane 5, Cys\(^{84}\)–Pro\(^{103}\). Each similar experiment was repeated three times, and representative results are shown.

Gln sites, a putrescine incorporation assay was performed using synthetic human MK. MK was incubated overnight with transglutaminase in the presence of \(^{14}\)C putrescine, and then the number of putrescine molecules incorporated into one molecule of MK was calculated from the radioactivity. The result is shown in Fig. 3 (column 1). About 2 mol of putrescine was incorporated/mol of MK, suggesting the presence of at least two different Gln sites in a molecule. The incubation time and the concentration of putrescine employed gave the maximum amount of putrescine incorporated as a function of both the putrescine concentration and the incubation time (data not shown). MK is structurally composed of two domains, called N1/2 (Lys\(^{1}\)–Gly\(^{29}\)) and C1/2 (Ala\(^{60}\)–Asp\(^{71}\), Ref. 15). To determine which domain contains the Gln sites, the incorporation of putrescine into synthetic N1/2 or C1/2 was measured. As can be
Pro\textsuperscript{51} peptide showed a complete inhibition against intact MK (panel A), N1/2 (panel B), and C1/2 (panel C). The Cys\textsuperscript{94}–Pro\textsuperscript{103} peptide also showed a complete inhibition toward the cross-linking of intact MK (panel A) and N1/2 (panel B), and partial inhibition against the cross-linking of C1/2 (panel C). The retardation and smear formation of each monomer observed in lanes 2 and 5 suggested that these peptides were preferentially cross-linked to molecules in question and, thereby, prevented cross-linking between monomer molecules. In this aspect, the results showed that Cys\textsuperscript{94}–Pro\textsuperscript{103} was incorporated into MK or its fragment molecules more slowly than Ala\textsuperscript{41}–Pro\textsuperscript{51}. Next, to determine which Gln residue in Ala\textsuperscript{41}–Pro\textsuperscript{51}, namely either Gln\textsuperscript{42} or Gln\textsuperscript{44}, participated in the cross-linking reaction, three mutant peptides were synthesized (Fig. 6); specifically, Gln\textsuperscript{42} was substituted with Ala, Gln\textsuperscript{44} was substituted with Ala, and both Gln\textsuperscript{42} and Gln\textsuperscript{44} were substituted with Ala, and then the competitive inhibition was tested. As shown in Fig. 7 (lane 2), the intact peptide was readily cross-linked to MK in a time-dependent manner and inhibited the cross-linking completely, consistent with the result in Fig. 5 (lane 2). Peptides in which one of the two Gln residues was substituted with Ala showed significant, but slower inhibition (lanes 3 and 4), suggesting that both Gln\textsuperscript{42} and Gln\textsuperscript{44} can serve as amine acceptor sites. In contrast, the double substituted peptide showed almost no inhibition (lane 5), suggesting that the inhibition by the peptides observed in lanes 2–4 was not due to the sequence structure other than Gln residues. Similar inhibition was observed with these peptides on the cross-linking of N1/2 or C1/2 (data not shown). In conclusion, Gln\textsuperscript{42} and Gln\textsuperscript{44} in the N1/2 domain as well as Gln\textsuperscript{95} in the C1/2 domain were suggested to function as potential amine-accepting sites in the transglutaminase-mediated cross-linking of MK.

Requirement of the Cross-linking of MK for Enhancement of the PA Level—Next, we examined whether or not the cross-linking of MK participated in the MK action of enhancing the PA level, using a Gln-containing peptide, Ala\textsuperscript{41}–Pro\textsuperscript{51}, that showed the strongest inhibition against the cross-linking reaction (Figs. 5 and 7). BAECs were incubated with MK with increasing amounts of Ala\textsuperscript{41}–Pro\textsuperscript{51}, and then the PA levels were determined. Indeed, the peptide suppressed the enhancement by MK (Fig. 8, curve B), whereas a mutant peptide, with double substitution of two Gln residues with Ala, did not (curve A). This suggested that interference of the cross-linking by Ala\textsuperscript{41}–Pro\textsuperscript{51} abolished the activity of MK added to the culture medium, namely that cross-linking of the MK monomer by cell surface transglutaminase might be required for the MK activity. If this speculation is correct and if the inhibition by the peptide only occurs at the step of the cross-linking, the once cross-linked dimer or tetramer should enhance the PA level without undergoing inhibition by the peptide. This was examined by means of an experiment in which the monomer, dimer,
FIG. 9. **PA-enhancing activity of the MK dimer.** Synthetic human MK (final: 3 μg) was incubated at 37 °C for 1 h with 1.25 μM tissue transglutaminase (in a total volume of 1.25 ml), in Hepes buffer containing 10 mM Ca^{2+} and 30 μg/ml heparin, and the reaction was terminated by the addition of 50 mM EDTA. Samples were concentrated 12.5-fold using an Ultrafree C3LG micro-concentrator (Millipore; molecular weight cut-off, 10,000) and then applied to a Superdex 75 HR 10/30 gel filtration column. The column was eluted with Hepes buffer containing 1 M NaCl, using a FPLC system, at the flow rate of 0.5 ml/min. The absorbance at 280 nm was monitored and shown as a dotted line (panel A). 0.5-ml fractions were collected, concentrated to 24 μl, and then either examined for PA-enhancing activity in the absence (closed circles) or presence (open circles) of 24 μg/ml (15.2 μM) Ala^{41–Pro^{51}} peptide, as described in Fig. 8 (panel A), or assessed the molecular species of MK by Western blotting (panel B). The PA activity levels plotted are averages (n = 3). Each similar experiment was repeated twice, representative results being shown.

and other multimers were separated by gel filtration chromatography, followed by assaying for PA-enhancing activity in the absence and presence of the Ala^{41–Pro^{51}} peptide (Fig. 9, panel A). The PA-enhancing activity (closed circles) was detected in fractions corresponding to the peak of absorbance at 280 nm (dotted line). From a comparison with the molecular standards used for the gel filtration chromatography and from the results of Western blotting of each fraction (panel B), three major peaks were determined as the monomer, the dimer, and a mixture of the tetramer and hexamer, from lower molecular weight fractions. As depicted by open circles, Ala^{41–Pro^{51}} suppressed the PA-enhancing activity only in the monomer fraction, i.e. not in the dimer or tetramer/hexamer fraction. This supports the hypothesis that transglutaminase-mediated cross-linking of the MK monomer is required for its ability to enhance the PA level in BAECs.

**Non-covalent Association of MK**—As can be seen in Fig. 9 (panel B), several bands were detected on Western blotting of the peak fractions. For example, the hexamer, tetramer, and dimer were seen in fraction 23, which was determined to be composed of the hexamer and tetramer, and the dimer and monomer were detected in fraction 26, which was determined to be the dimer. As a reason for this, we speculated that MK associated non-covalently in addition to the covalent cross-linking by transglutaminase. To determine if such a non-covalent association really occurs, we performed the cross-linking reaction in the absence of free Ca^{2+}, and then compared the chromatograms obtained on FPLC (Fig. 10). In the presence of free Ca^{2+}, namely when transglutaminase functioned, almost the same amounts of the monomer, the dimer, and the tetramer/hexamer mixture were detected in fractions 36, 26, and 23, respectively (curve B, a solid line). However, even when transglutaminase was blocked by chelating Ca^{2+}, almost the same peak was detected for fraction 26, and about a half amount of the peak was detected for fraction 23, in addition to a peak of the monomer for fraction 36 (curve A, a dashed line). Furthermore, the PA-enhancing activity was detected in accordance with the peak of multimers in addition to monomer (Fig. 11). Western blots of these fractions only showed the monomer (data not shown), indicating that the peaks detected for fractions 26 and 23 represented the non-covalently associated MK dimer, tetramer, or hexamer, which dissociated upon exposure to SDS. Therefore, under the current experimental conditions, MK forms multimers via two distinct mechanisms, transglutaminase-mediated covalent cross-linking and non-covalent association. We next examined the effect of Ala^{41–Pro^{51}} on this non-covalent association. The cross-linking reaction was performed in the presence of an excess of the peptide, and multimer formation was assessed by gel filtration chromatography. As depicted by curve C (a double-dashed line) in Fig. 10, almost no peak was detected for fraction 26, although two small peaks were detected for fractions 22 and 23, suggesting that not only the cross-linking, but also the non-covalent association was suppressed by the Ala^{41–Pro^{51}} peptide.

**DISCUSSION**

Retinol (vitamin A) and its derivatives (retinoids) have profound effects on the regulation of cell growth and differentiation (44). Using BAEC cultures, we found that retinoids induce the production of PA (45), transglutaminase (46), transforming growth factor-β (TGF-β; Ref. 47), and MK (22), and that these retinoic acid-inducible factors interact with each other. In retinoic acid-treated BAECs, PA and transglutaminase are required to promote the activation of latent TGF-β (11, 47), whereas MK and TGF-β regulate PA activity, respectively, in the opposite way (11, 22). The present paper describes an additional relationship between transglutaminase and MK.

MK was proved to be an excellent substrate for transglutaminase in that 1 mol of MK incorporated as much as 2 mol of [1^{14}C]putrescine. MK formed the dimer, tetramer, and hexamer on incubation with transglutaminase. Among multimers dimer seemed to be dominant. Whereas the results in Figs. 5 and 7 suggest that Gln^{42} and Gln^{44} in N1/2 as well as Gln^{95} in C1/2 may function as potential amine-accepting sites, the results in Fig. 3 suggest the existence of one Gln site in each of N1/2 and
C1/2. The most likely explanation for this difference is that although both Gln\(^{42}\) and Gln\(^{44}\) are competent enough to serve as amine-accepting sites, only one of them is exposed to the surface of the MK molecule. The second likely explanation is that Gln\(^{42}\) and Gln\(^{44}\) serve heterogeneously as amine-accepting sites. Namely, when Gln\(^{42}\) is used to form an e-(γ-glutamyl)lysine bridge between a Lys residue in another MK molecule, or putrescine in the present experiment, neighboring Gln\(^{44}\) becomes inaccessible for further bridge formation due to stereo-inhindrance by the MK molecule cross-linked to Gln\(^{42}\). Conversely, when Gln\(^{44}\) is cross-linked to another MK molecule, Gln\(^{42}\) becomes no longer accessible. Stereo-inhindrance will also happen when Factor XIIIa is used as the enzyme. As Factor XIIIa is larger than tissue transglutaminase (\(\sim 300 \text{kDa versus } 80 \text{kDa}\)), the cross-linking of MK molecules may not be performed by Factor XIIIa, even though it can stimulate putrescine incorporation into MK molecules. It is notable that these three acyl-donating Gln residues are conserved among the MK/PTN family, whereas the other two Gln residues are not (48). As can be seen in Fig. 4, the dimer and tetramer of MK C1/2 migrated slower than those of N1/2. This might be due to that C1/2 is much more highly charged being basic. The three-dimensional structure of MK C1/2 recently clarified by NMR spectroscopy\(^2\) is consistent with the results of the present investigation. In C1/2, basic amino acids, which are expected to form the heparin binding site (Arg\(^{81}\), Lys\(^{86}\), and Lys\(^{87}\)), are clustered on one side. Site-directed mutagenesis of these amino acids resulted in decreased heparin-binding and neurite-promoting activities.\(^3\) Gln\(^{95}\), which was shown to be involved in the dimerization, is located on the opposite side. This distinct localization of the heparin binding site and the cross-linking site will permit the cross-linking between two MK molecules after binding to heparin. Thus, heparin may potentiate the cross-linking with transglutaminase by stabilizing the conformation of dimer. In addition, heparin increases the amount of products, too, by preventing the loss of MK molecules from the reaction mixture that happens due to static adherence of MK molecules to the vessel wall (43). Because of this reason and because we supposed the physiological reaction on the cell surface as discussed below, we analyzed the dimer formation in the presence of heparin. We are currently trying to determine the amine-donating Lys sites in the MK molecule. It is also of great importance to clarify the structural requirements for the substrate for transglutaminase, i.e. the role of flanking sequences in the Ala\(^{41}\)-Pro\(^{53}\) peptide, and to compare it with the result obtained with fibronectin-derived sequence (49).

We have concluded that dimerization of MK by cell surface transglutaminase potentiates MK activity, based upon the fact that the Ala\(^{41}\)-Pro\(^{53}\) peptide inhibits both the cross-linking and PA-enhancing activity of the MK monomer, with a double mutated peptide as an inactive control. We did not obtain additional proof utilizing an anti-transglutaminase antibody, because the antibody could inhibit the formation of TGF-β, which counteracts MK activity (22). Since the activation of latent TGF-β is required for transglutaminase to localize latent TGF-β on the surface, the inclusion of an anti-transglutaminase antibody in the culture medium prevents the formation of active TGF-β (11). Hence, the peptide was a strong tool to prove our hypothesis, although the specificity of the inhibition by the peptide was critical to obtain a conclusion. As a receptor interacting site(s) is located in C1/2 (37, 38), there is a low possibility that the Ala\(^{41}\)-Pro\(^{53}\) peptide competes with the binding of MK to its receptor(s). Actually, the dimer exerted the PA-enhancing activity in the presence of this peptide. As Factor XIIIa functions only in putrescine incorporation, it is possible that Factor XIIIa might be used to cross-link peptide to MK molecule, serving as a control for this issue. It is true that when the PA-enhancing effect of the MK monomer is blocked by Ala\(^{41}\)-Pro\(^{53}\), cross-linking of the MK monomer, especially formation of the dimer, is suppressed completely. However, it is possible that other transglutaminase-mediated cross-linking reactions were affected by the Ala\(^{41}\)-Pro\(^{53}\) peptide, and that this caused the inhibition of MK activity by the peptide. In this context, we need to examine the specificity of the inhibition by this peptide. Although the peptide inhibited the cross-linking of intact MK, N1/2, and C1/2 by 80% at a concentration of as much as a 50-fold molar excess, a 200-fold molar excess of this peptide caused only weak (less than 5%) inhibition of the cross-linking of pro-SPAI (data not shown; Ref. 10). We are now investigating whether the peptide affects the cross-linking of other hitherto known substrates for transglutaminase. Nevertheless, we believe that the present conclusion is correct, because in the experiment in Fig. 9, whether an inhibitory effect of the peptide was observed

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\(^2\) F. Inagaki, personal communication.
\(^3\) N. Asai, unpublished result.
and, now, MK.

cross-linking of cytokines such as TGF-
plays a role in the regulation of cellular physiology through the hypothesisis concerning the mechanism whereby transglutaminase its unidentified receptors by making them form the dimer. An
cytokine and hormone receptors is important for the emission of Recently, it was reported that hetero- or homodimerization of
sized MK is cross-linked before secretion or that secreted MK is

cross-linked on the cell surface as discussed above. The results in
Fig. 2 suggest that the latter reaction may proceed very effi-
ciently with the aid of heparan sulfate present on the cell surface. Tor XIIIa is not able to cross-link MK molecules and tissue
formation alone is sufficient to stimulate increases in PA-en-
hancing activity in the dimer fraction (Fig. 9). Once non-
covalent association occurs between MK molecules, the cross-
linking is exposed. Figs. 9 and 11 suggest that dimer/oligomer
formation alone is sufficient to stimulate increases in PA-en-
hancing activity and that binding of MK to cells via cell surface
transglutaminase is not necessary. However, since plasma Fact-
or XIIIa is not able to cross-link MK molecules and tissue
transglutaminase is not released from the cells (46), the surface
reaction may be physiologically most important. The suscepti-
bility of MK to transglutaminase suggests a mechanism whereby
the interaction of MK with surface receptors and other surface-
oriented structures could be enzymatically altered. Tissue trans-
glutaminase is distributed mainly intracellularly and partially
on the cell surface (11, 46), suggesting either that newly synthe-
sized MK is cross-linked before secretion or that secreted MK is
cross-linked on the cell surface as discussed above. The results in
Fig. 2 suggest that the latter reaction may proceed very effi-
ciently with the aid of heparan sulfate present on the cell surface. Recently, it was reported that hetero- or homodimerization of
cytokine and hormone receptors is important for the emission of signals inside cells (50, 51). Together with the finding that the cross-linking is important for PA-enhancing activity, we imagine that the MK dimer, cross-linked on the cell surface, may activate its unidentified receptors by making them form the dimer. An experiment addressing this hypothesis is in progress.

The current study provided additional evidence for the hypo-
thesis concerning the mechanism whereby transglutaminase plays a role in the regulation of cellular physiology through the cross-linking of cytokines such as TGF-β (11), interleukin 2 (12) and, now, MK.

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REFERENCES

1. Lorand, L., and Conrad, S. M. (1984) Mol. Cell. Biochem. 58, 9–35
2. Greenberg, C. S., Birckbichler, P. J., and Rice, R. H. (1991) FASEB J. 5, 3071–3077
3. Gentile, V., Saydk, M., Gioia, E. A., Aikande, O., Birckbichler, P. J., Lee, K. N., Stein, J. P., and Davies, P. J. A. (1991) J. Biol. Chem. 266, 478–483
4. Nakayoshi, K., Naka, K., Hagiwara, H., Aoyama, Y., Ueno, H., and Hirose, S. (1991) J. Biol. Chem. 266, 15,212–15,215
5. Lu, S., Saydk, M., Gentile, V., Stein, J. P., and Davies, P. J. A. (1995) J. Biol. Chem. 270, 9748–9756
6. Thomazy, V., and Pitsis, L. (1989) Cell Tissue Res. 255, 215–224
7. Floyd, E. E., and Jetten, A. M. (1989) Mol. Cell. Biol. 9, 4846–4851
8. Marvin, K. W., George, M. D., Fujimoto, W., Saunders, N. A., Bernacki, S. H., and Jetten, A. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 11036–11030
9. Bendixen, B., Berth, W., and Harper, P. C. (1993) J. Biol. Chem. 268, 21692–21697
10. Nara, K., Ito, S., Ito, T., Suzuki, Y., Gheonime, M. A., Tachibana, S., and Hirose, S. (1994) J. Biol. Chem. 269, 441–448
11. Kojima, S., Nara, K., and Rifkin, D. B. (1993) J. Cell Biol. 121, 439–448
12. Eitan, S., and Schwartz, M. (1993) Science 261, 106–108
13. Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Hossain, A., Misori, K., Im, M.-J., and Graham, R. K. (1994) Science 264, 1593–1596
14. Zhang, L.-X., Mills, K. J., Dawson, M. I., Collins, S. J., and Jetten, A. M. (1995) J. Biol. Chem. 270, 6022–6029
15. Muramatsu, T. (1994) Dev. Growth Factors 6, 1–8
16. Kurtz, A., Schutte, A. M., and Wellstein, A. (1995) Crit. Rev. Oncogen. 6, 151–177
17. Kadomatsu, K., Tomomura, M., and Muramatsu, T. (1988) Biochem. Biophys. Res. Commun. 151, 1312–1318
18. Muramatsu, H., and Muramatsu, T. (1991) Biochem. Biophys. Res. Commun. 177, 652–658
19. Nurembe, V., Fraser, N., Herlaar, E., and Heath, J. K. (1992) Development 116, 1175–1183
20. Nakanishi, K., Nara, K., Hagiwara, H., Aoyama, Y., Ueno, H., and Hirose, S. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 679–682
21. Tomomura, M., Kadomatsu, K., Matusaba, S., Nakagawara, A., Hamaeoue, M., Takao, S., Shimazu, H., Ohi, Y., and Muramatsu, T. (1993) Cancer Res. 53, 1281–1285
22. Floyd, E. E., and Jetten, A. M. (1989) Cell Tissue Res. 265, 10765–10770
23. Fabri, L., Nice, E. C., Wu, D. H., Maruta, H., Burgess, A. W., and Simpson, R. J. (1998) Biochem. Int. 119, 1267–1271
24. Fabri, L., Maruta, H., Muramatsu, H., Muramatsu, T., Simpson, R. J., Burgess, A. W., and Nice, E. C. (1993) J. Chromatogr. 646, 213–225
25. Muramatsu, H., Inui, T., Kimura, T., Sakakibara, S., Song, X., Maruta, H., and Muramatsu, T. (1994) Biochem. Biophys. Res. Commun. 203, 1131–1139
26. Kojima, S., Inui, T., Kimura, T., Sakakibara, S., Song, X., Maruta, H., and Muramatsu, T. (1995) Biochem. Biophys. Res. Commun. 206, 468–473
27. Muramatsu, H., Shirahama, H., Yonezawa, S., Maruta, H., and Muramatsu, T. (1993) Dev. Biol. 159, 392–402
28. Perry, M. J. M., Mahoney, S.-A., and Haynes, L. W. (1995) Neurosci 65, 1063–1076
29. Mahoney, S.-A., Perry, M., Seddon, A., Bohlen, P., and Haynes, L. (1996) Biochem. Biophys. Res. Commun. 222, 154–157
30. Inui, T., Bodi, J., Kubo, S., Nishio, H., Kimura, T., Kojima, S., Maruta, H., Muramatsu, T., and Sakakibara, S. (1996) J. Peptide Sci. 2, 28–39
31. Inui, T., Kojima, S., Muramatsu, H., Kimura, T., Sakakibara, S., and Muramatsu, T. (1995) Biochem. Biophys. Res. Commun. 216, 574–581
32. Gudas, L. J., Sporn, M. B., and Roberts, A. B. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) 2nd Ed., pp. 443–520, Raven Press, New York
33. Kriches, I. J., and Henders, H., and Graham, R. K., and Schuening, W.-D. (1983) Gene (Amst.) 125, 177–183
34. Naka, K., Nakashiki, K., Hagiwara, H., Waki, K., Kojima, S., and Hirase, S. (1996) J. Biol. Chem. 271, 19308–19312
35. Kojima, S., and Rifkin, D. B. (1993) J. Cell Biol. 155, 323–332
36. Sekiguchi, K., Yokota, C., Asashima, M., Kaname, T., Fan, Q.-W., Muramatsu, T., and Kadomatsu, K. (1995) J. Biochem. (Tokyo) 118, 84–99
37. Muramatsu, K., Velasco, P. T., Wilson, J., and Lorand, L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1–6

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