The Journal of Biological Chemistry
Vol. 273, No. 9, Issue of February 27, pp. 4815–4818, 1998
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Communication

A Pentacosapeptide (CKS-25) Homologous to Retroviral Envelope Proteins Possesses a Transforming Growth Factor-β Activity*

(Received for publication, October 8, 1997, and in revised form, December 23, 1997)

Shuan Shian Huang and Jung San Huang‡

From the Department of Biochemistry and Molecular Biology, St. Louis University School of Medicine, St. Louis, Missouri 63104

CKS-17, a synthetic heptadecapeptide homologous to a conserved domain in retroviral envelope protein p15E, mimics the immunosuppressive properties of p15E in vitro and in vivo, but the mechanisms are not understood. Here we report that a synthetic pentacosapeptide designated CKS-25, a longer version of CKS-17 that contains a functional transforming growth factor-β (TGF-β) active-site motif (RXXD), inhibits 125I-labeled TGF-β1 (125I-TGF-β1) binding to cell-surface TGF-β receptors in cultured epithelial cells. Multiple conjugation of CKS-25 to bovine serum albumin and carbonic anhydrase enhances the 125I-TGF-β1 binding inhibitory activity and confers a partial TGF-β agonist activity (growth inhibition but not transcriptional activation). Since TGF-β is a potent immunosuppressive factor, these results suggest that the immunosuppressive properties of CKS-17-bovine serum albumin conjugate and p15E are mediated at least in part by their TGF-β agonist activities.

Although retroviral infections frequently cause immunosuppression, the molecular mechanisms of retrovirus-induced immunosuppression are not understood (1, 2). Increasing evidence suggests that p15E, the transmembrane envelope protein of feline and murine leukemia viruses, possesses immunosuppressive activities (1, 2). A hydrophilic 26-amino acid region of the p15E envelope protein is conserved among the transmembrane envelope proteins of animal and human retroviruses, including human T-cell leukemia virus and human immunodeficiency virus (1–4). CKS-17, a heptadecapeptide whose amino acid sequence is derived from this conserved region, is immunosuppressive in vitro and in vivo after conjugation with bovine serum albumin (BSA)1 (1–4). The mechanisms by which p15E and CKS-17-BSA conjugate cause immunosuppression are not known (1, 2). Here we show that CKS-25, a pentacosapeptide and a longer version of CKS-17 that possesses a putative TGF-β1 active-site motif (RXXD) (5), inhibits 125I-labeled TGF-β1 binding to cell-surface TGF-β receptors. Evidence is presented that the RXXD motif is essential for the inhibition of TGF-β binding. We also demonstrate that CKS-25-BSA and CKS-25-carbonic anhydrase (CA) conjugates possess a TGF-β agonist activity.

EXPERIMENTAL PROCEDURES

Materials—Na125I (17 Ci/mg) and [methyl-3H]thymidine (67 Ci/mmol) were purchased from ICN Biomedical, Inc. (Costa Mesa, CA). High molecular mass protein standards (myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase, 97 kDa; bovine serum albumin, 66 kDa), BSA, human CA, and other chemical reagents were obtained from Sigma. Disuccinimidyl suberate (DSS) was obtained from Pierce. Human TGF-β1 was purchased from Austral Biologicals (San Ramon, CA). β2M (41–65), a specific TGF-β peptide antagonist whose amino acid sequence is derived from and corresponds to the 41st to 65th amino acid residues of TGF-β1, was prepared as described previously (5). Mink lung epithelial cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum.

Preparation of CKS-25, CKS-25 RA/DA, and CKS-25 Protein Conjugates—CKS-25, a pentacosapeptide whose amino acid sequence corresponds to the 62nd to 86th amino acid residues of a murine leukemia virus (AKV) (6), and CKS-25 RA/DA, in which both arginine and aspartic acid residues in the RXXD motif are replaced with alanine residues, were synthesized using tert-butoxycarbonyl chemistry on an Applied Biosystems model 431A peptide synthesizer and purified by reverse-phase high pressure liquid chromatography (7). The purity (>95%) of the synthetic peptides was estimated by automated Edman degradation. CKS-25-BSA and CKS-25-CA conjugates were prepared using DSS as the conjugation agent at a molar ratio of DSS:BSA(or CA):peptide (270:8:450) (5). CKS-25-BSA and CKS-25-CA conjugates contained 5–10 peptides per molecule of protein and were used throughout the experiments. CKS-25-BSA and -CA, which were prepared at a molar ratio of DSS:BSA (or CA):peptide (27:8:45) contained 1–2 peptides per molecule of protein and were not significantly more active than CKS-25 without conjugation.

125I-TGF-β1 Binding and Affinity Labeling in Mink Lung Epithelial Cells—The 125I-TGF-β1 binding and affinity labeling were carried out as described previously (8). The specific binding of 125I-TGF-β1, was estimated by subtracting nonspecific binding from total binding. The nonspecific binding was obtained in the presence of 100-fold excess of unlabeled TGF-β1. The 125I-TGF-β1 affinity-labeled cell-surface TGF-β receptors were analyzed by 5% SDS-polyacrylamide gel electrophoresis and autoradiography.

[methyl-3H]Thymidine Incorporation and RNA Analysis—Cells grown on 24-well cluster dishes at near confluence were incubated with various concentrations of CKS-25, CKS-25-BSA, or CKS-25-CA in the presence of several concentrations of TGF-β1 or 10 μM β2M (41–65) (5) in Dulbecco’s modified Eagle’s medium containing 0.1% fetal calf serum. The assay of [methyl-3H]thymidine incorporation into cellular DNA was carried out in triplicate as described previously (9). For RNA analysis, cells grown on 12-well cluster dishes were treated with 10 μM CKS-25, 10 μM CKS-25-BSA, 10 μM CKS-25-CA conjugate, or TGF-β1 (0.25 and 2.5 pM) at 37 °C for 2.5 h in 0.1% fetal calf serum. The extraction of RNA and Northern blot analysis were carried out as described previously (9).

RESULTS AND DISCUSSION

We have recently developed three potent synthetic TGF-β1 pentacosapeptide antagonists (β1m(41–65), β2M(41–65), and β2M(41–65)), which all contain a (W/R)XXD motif that is essential for their TGF-β antagonist activities (5). Conjugates containing multiple units of these pentacosapeptides conjugated to BSA or CA have a partial TGF-β agonist activity (5). These results led us to investigate the TGF-β activity of immunosuppressive retroviral envelope proteins that contain this...
Fig. 1. Amino acid sequences for the conserved region of retrovirus transmembrane envelope proteins and of human TGF-β1, TGF-β2, and TGF-β3 peptide antagonists. The putative TGF-β active-site motif is underlined. Human immunodeficiency virus, type 1 gp41 contains two motifs (RLAE and LAVE). CKS-25, a synthetic retroviral envelope pentacapeptide; CKS-17, a synthetic retroviral envelope heptadecapeptide; β2, a synthetic TGF-β1 peptide antagonist with an amino acid sequence that corresponds to the 41st to 65th amino acid residues of human TGF-β1; β2(41–65), a synthetic TGF-β2 peptide antagonist with an amino acid sequence corresponding to the 41st to 65th amino acid residues of human TGF-β2; β3(41–65), a synthetic TGF-β3 peptide antagonist with amino acid sequence corresponding to the 41st to 65th amino acid residue of human TGF-β3; MoMuLV, Moloney murine leukemia virus; FLV, Friend leukemia virus; AKV, a murine leukemia virus endogenous to various strains of mice, e.g. AKR; GLV, Gross leukemia virus; MMCp, mink-cell focus-forming virus; AMCF, mink-cell focus-forming virus of AKR origin; FeLV, feline leukemia virus; BLV, bovine leukemia virus; MPMV, Mason-Pfizer monkey virus; SRV, simian retrovirus; HTLV-1, human T-cell leukemia virus type 1; HTLV-2, human T-cell leukemia virus type 2; HIV-1, human immunodeficiency virus, type 1.

Fig. 2. Inhibition of 125I-TGF-β1 binding (A) and affinity labeling (B) in mink lung epithelial cells by CKS-25 and CKS-25-BSA (or -CA). Cells were incubated with 0.1 nM 125I-TGF-β1 in the presence of various concentrations of CKS-25 or CKS-25-BSA (or -CA). The specific binding of 125I-TGF-β1 and 125I-TGF-β3-affinity labeling was then determined. The specific binding of 125I-TGF-β1, in the absence of CKS-25 and CKS-25-BSA (-CA) was taken as 0% inhibition (12,591 ± 981 cpm/well). The error bars were derived from the standard deviation of triplicate cell cultures. The figure is representative of seven experiments, which have comparable results. 125I-TGF-β1-affinity labeling of cell-surface TGF-β receptors was analyzed by 5% SDS-polyacrylamide gel electrophoresis and autoradiography. The brackets indicate the location of types 1, II, and III TGF-β receptors (TβR-I, TβR-II, and TβR-III). The arrow indicates the location of the type V TGF-β receptor (TβR-V).

The putative TGF-β3 active-site motif (RXAD) and have oligomeric structures (1, 2). As shown in Fig. 1, the amino acid sequences for a conserved region of retrovirus transmembrane envelope proteins contain a RGLD motif (1, 2). This is homologous to the motif (RSAD) of β2(41–65) peptide, the amino acid sequence of which corresponds to the 41st to 65th amino acid residues of human TGF-β1 (5). The other motif (LAWE) in human immunodeficiency virus, type 1 gp41 is homologous to those of β2(41–65) and β2(41–65) peptides (WSLD and WSAD) (5). The amino acid sequences of β2(41–65) and β3(41–65) peptides correspond to a motif of α-helix in the three-dimensional structure of p15E peptide fragment, its configuration in the structure of intact p15E molecule is not established (13, 14). However, it seems likely that the side chains of the arginine and aspartic acid residues in the RGLD motif of p15E protein are exposed to solvents (11, 12). Although the RGLD motif is located at the end of an α-helix in the three-dimensional structure of a p15E peptide fragment, its configuration in the structure of intact p15E molecule is not established (13, 14).

To test the TGF-β antagonist activity of the immunosuppressive retroviral envelope proteins (i.e. their ability to block TGF-β binding to the TGF-β receptors), we synthesized a pentacapeptide, designated CKS-25, whose amino acid sequence is derived from the viral p15E protein of the AKV murine leukemia virus. It corresponds to amino acid residues 62–86 of the viral protein (6). CKS-25 includes the whole amino acid sequence of CKS-17 and has additional amino acid residues at the N and C termini of CKS-17 (Fig. 1). CKS-17 has been shown to comprise the active-site sequence of p15E (1, 2). The TGF-β antagonist activity of CKS-25 was analyzed by incubating mink lung epithelial cells with 0.1 nm 125I-TGF-β1 in the presence of various concentrations of CKS-25 at 0 °C for 2.5 h. As shown in Fig. 2A, CKS-25 at 75 µM inhibited about 50% of 125I-TGF-β1 binding to cell-surface TGF-β receptors of mink lung epithelial cells. This IC50 (~75 µM) is comparable with that (~20 µM) of the peptide antagonist, β2(41–65), which contains the putative TGF-β3 active-site motif (RSAD) (5). Conjugates containing multiple peptides (~5–10 CKS-25 peptides per molecule of protein) conjugated to BSA and CA showed enhanced 125I-TGF-β binding inhibitory activity (IC50 of ~80–150 nm). The analysis of 125I-TGF-β1 affinity labeling of cell-surface TGF-β receptors revealed that CKS-25-BSA conjugate (1 µM) almost completely abolished 125I-TGF-β1 binding to all cell-surface TGF-β receptors including types I, II, III, and V TGF-β receptors (Fig. 2B, lane 1 versus lane 2). To investigate the importance of the RGLD motif in the activity of CKS-25, a structural variant of CKS-25 designated CKS-25 RA/DA (in which the arginine and aspartic acid residues in the motif were replaced with alanine residues) was synthesized and tested for its 125I-TGF-β3 binding inhibitory activity. CKS-25 RA/DA showed no inhibition of 125I-TGF-β3 binding to cell-surface TGF-β receptors in mink lung epithelial cells (Fig. 2A). These results suggest that the RXXD motif in CKS-25 is important for its activity.

TGF-β is a potent growth inhibitor for a variety of cell types including epithelial cells, endothelial cells, and T-cells (2, 15, 16). To determine the TGF-β agonist and antagonist activities of CKS-25 and CKS-25-protein conjugates, we determined their effects on the growth of mink lung epithelial cells as measured by [methyl-3H]thymidine incorporation into cellular DNA. As shown in Fig. 3A, CKS-25 (10 µM) blocked the inhibition of [methyl-3H]thymidine incorporation into cellular DNA induced by 0.25 µM TGF-β1. In the absence of TGF-β1, CKS-25 did not affect DNA synthesis. By contrast, the protein conjugates of CKS-25, CKS-25-BSA, and CKS-25-CA inhibited DNA synthesis ~40–50% in mink lung epithelial cells at 1 µM (Fig. 3B). The CKS-25-BSA-induced DNA synthesis inhibition was blocked in the presence of 10 µM β2(41–65), a specific TGF-β3 peptide antagonist, suggesting that the inhibition is mediated by TGF-β2 receptors (Fig. 3B). β2(41–65) also blocked the CKS-25-CA-induced DNA synthesis inhibition (data not shown), and both BSA and CA conjugated without peptide did not have...
growth inhibitory activity as reported previously (5). These results indicate that multiple conjugation of CKS-25 peptides to proteins produces a multivalent ligand that has TGF-β activity inducing growth inhibitory response though much higher concentrations are required to exhibit this activity (IC₅₀ = 0.1–0.3 μM).

The transactivation of plasminogen activator inhibitor 1 (PAI-1) and fibronectin is the other characteristic response of TGF-β binding to receptors (15, 16). Thus, we investigated the effect of CKS-25-BSA on the transcriptional expression of PAI-1. At 10 μM, CKS-25-BSA did not have a significant effect on PAI-1 expression (Fig. 4), indicating that CKS-25-BSA acts as an agonist in the growth inhibition assay but not in the assay that measures transcriptional regulation of TGF-β receptors (15). Nonetheless, CKS-25-BSA could compete for binding to the TGF-β receptors and function as an antagonist for TGF-β-induced PAI-1 expression (Fig. 4).

An interesting question raised by the growth inhibitory activities of CKS-25-protein conjugates reported here is which if any of the known TGF-β receptor types mediate their growth inhibitory activities. Mink lung epithelial cells, T-cells, and other normal cell types express types I, II, III, and V TGF-β receptors (7, 8, 17, 18). The types I, II, and V TGF-β receptors are Ser/Thr-specific protein kinases and mediate signaling that leads to cellular responses (7, 8, 17, 18). The type III TGF-β receptor is a proteoglycan membrane protein, which is believed not to participate directly in signaling (17, 18). The type V TGF-β receptor has been recently shown to mediate a growth inhibitory response though the types I and II TGF-β receptors are required for the maximal growth inhibitory response (8). The CKS-25 monovalent ligand competes for TGF-β binding to all the receptors. It is thus possible that the growth inhibitory response stimulated by CKS-25 protein conjugates could be mediated by any of the known receptors or some combination. The type V TGF-β receptor was suggested as a candidate by the finding that insulin-like growth factor protein 3, which also contains a WCVD motif (19), is a specific ligand for the type V TGF-β receptor and exhibits partial TGF-β agonist activity (growth inhibition but not transcription activation) (10). Like the CKS-25 protein conjugates, insulin-like growth factor protein 3 exerts a maximum of ~50% inhibition of DNA synthesis (10).

Since TGF-β is one of the most potent immunosuppressive polypeptides known (2) and since we show here that CKS-25 protein conjugates can function as partial TGF-β agonists, we hypothesize that the transmembrane envelope protein p15E of retroviruses suppresses immune responses at least in part by their TGF-β agonist activities. This hypothesis is supported by several lines of evidence. 1) Although TGF-β is more potent than p15E and CKS-17-BSA conjugate, p15E, CKS-17-BSA conjugate, and TGF-β have similar immunosuppressive activities (2) CKS-17 or CKS-25 comprises the active-site sequence of p15E (1, 2). 3) CKS-25 and CKS-25-protein conjugates compete with 125I-TGF-β for binding to cell-surface TGF-β receptors (Fig. 2). 4) CKS-25-protein conjugates by themselves exhibit growth inhibitory activities that can be blocked by a monovalent TGF-β-specific peptide antagonist, β2(41–65) (Fig. 3). 5) Multivalency appears to be required for the TGF-β agonist activity. Similarly, it is known that the dimeric structure of TGF-β is required for its biological activities (11, 12). The heterodimerization or hetero-oligomerization of the TGF-β receptors is known to be important for the biological responses to TGF-β (20). Also, p15E is a trimeric protein and present in many copies in the viral envelope (1, 2, 13). CKS-17-BSA/CKS-25-BSA has multiple valences of the putative active-site motif (1, 2, 5). The multivalency of all of these structures may allow them to activate TGF-β receptors by inducing heterodimerization or hetero-oligomerization, which...
can explain their TGF-β agonist-like activity in a growth inhibition assay system.

An interesting and unresolved question is how these multivalent conjugates show selectivity in their TGF-β agonist activity. It will be very interesting to determine how the TGF-β receptors respond selectively with the growth inhibition response without inducing the other prominent response, producing a significant effect on transcriptional regulation of PAI-1.

Acknowledgments—We thank Drs. William S. Sly and Frank E. Johnson for critical review of the manuscript. We also thank John H. McAlpin for typing the manuscript.

REFERENCES
1. Haraguchi, S., Good, R. A., and Day, N. K. (1995) Immunol. Today 12, 595–602
2. Oostendorp, R. A. J., Meijer, C. J. L., and Scheper, R. J. (1993) Rev. Oncol./Hematol. 14, 189–206
3. Haraguchi, S., Good, R. A., James-Yarish, M., Cianciolo, G., and Day, N. K. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3611–3615
4. Denner, J., Norley, S., and Kurth, R. (1994) AIDS 8, 1063–1071
5. Huang, S. S., Liu, Q., Konish, Y., Johnson, F. E., and Huang, J. S. (1997) J. Biol. Chem. 272, 27155–27159
6. Elder, J. H., and Mullins, J. I. (1983) J. Virol. 46, 871–880
7. Liu, Q., Huang, S. S., and Huang, J. S. (1994) J. Biol. Chem. 269, 9221–9226
8. Liu, Q., Huang, S. S., and Huang, J. S. (1997) J. Biol. Chem. 272, 18891–18895
9. O'Grady, P., Liu, Q., Huang, S. S., and Huang, J. S. (1992) J. Biol. Chem. 269, 21033–21037
10. Leal, S. M., Liu, Q., Huang, S. S., and Huang, J. S. (1997) J. Biol. Chem. 272, 20572–20576
11. Schlunegger, M. P., and Grueter, M. G. (1992) Nature 358, 430–434
12. Hinck, A. P., Archer, S. J., Qian, S. W., Roberts, A. B., Sporn, M. B., Weatherbee, J. A., Tsang, M. L.-S., Lucas, R., Zhang, B.-L., Wenker, J., and Torchia, D. A. (1996) Biochemistry 35, 8537–8544
13. Fass, D., Harrison, S. C., and Kim, P. S. (1995) Nat. Struct. Biol. 3, 465–469
14. Weissenhorn, W., Dessen, A., Harrison, S. C., Skehel, J. J., and Wiley, D. C. (1997) Nature 387, 426–430
15. Roberts, A. B., and Sporn, M. B. (1991) in Peptide Growth Factors and Their Receptors (Sporn, M. B., and Roberts, A. B., eds) pp. 410–472, Springer-Verlag, Heidelberg, Germany
16. Massagué, J. (1990) Annu. Rev. Cell Biol. 6, 597–641
17. Yingling, J. M., Wang, X.-F., and Bassing, L. H. (1995) Biochim. Biophys. Acta 1242, 115–130
18. Massagué, J. (1992) Cell 69, 1067–1070
19. Martin, J. L., Ballesteros, M., and Baxter, R. C. (1993) Endocrinology 131, 1703–1710
20. Wrana, J. L., Attisano, L., Wierser, R., Ventura, F., and Massagué, J. (1994) Nature 370, 341–346
A Pentacosapeptide (CKS-25) Homologous to Retroviral Envelope Proteins Possesses a Transforming Growth Factor-β Activity
Shuan Shian Huang and Jung San Huang

J. Biol. Chem. 1998, 273:4815-4818.
doi: 10.1074/jbc.273.9.4815

Access the most updated version of this article at http://www.jbc.org/content/273/9/4815

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 19 references, 6 of which can be accessed free at
http://www.jbc.org/content/273/9/4815.full.html#ref-list-1