Deletion of the Carboxyl Terminus of Tie2 Enhances Kinase Activity, Signaling, and Function

EVIDENCE FOR AN AUTOINHIBITORY MECHANISM*

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Tie2 is an endothelial receptor tyrosine kinase that is required for both embryonic vascular development and tumor angiogenesis. There is considerable interest in understanding the mechanisms of Tie2 activation for therapeutic purposes. The recent solution of the Tie2 crystal structure suggests that Tie2 activity is autoinhibited by its carboxyl terminus. Here we investigated the role of the C tail in Tie2 activation, signaling, and function both in vitro and in vivo by deleting the C terminus of Tie2 (ΔCT). Compared to wild type Tie2, in vitro autophosphorylation and kinase activity were significantly enhanced by the ΔCT mutation. In NIH 3T3 cells expressing chimeric Tie2 receptors, both basal and ligand-induced tyrosine phosphorylation were markedly enhanced compared to wild type in several independent clones of Tie2-ΔCT. Moreover, the ΔCT mutation enhanced basal and ligand-dependent activation of Akt and extracellular signal-regulated kinase. Enhanced Akt activation correlated with significant inhibition of staurosporine-induced apoptosis. These findings demonstrate that the Tie2 C tail performs a novel negative regulatory role in Tie2 signaling and function, and they provide important insights into the mechanisms by which the Tie2 kinase is activated.

Tie2/Tek is a receptor tyrosine kinase (RTK) that is expressed almost exclusively on endothelial cells and is required for both normal embryonic vascular development and tumor angiogenesis. Tie2 is unique among RTKs in that its ligands, the angiopoietins, have apparently opposing actions on Tie2 signaling and function. The activating ligand angiopoietin-1 (Ang1) potently blocks increases in vascular permeability induced by inflammatory agents and vascular endothelial growth factor (2–4). Tie2 activation is distinct from that of other RTKs, in which the activation loop (A-loop) obstructs substrate or ATP binding in the inactive state, the A-loop of Tie2 assumes an "active-like" conformation, which would appear to facilitate substrate binding. However, the nucleotide binding loop adopts an apparent inhibitory conformation, with the side chains of several residues in this loop occupying the ATP binding site. An additional level of enzyme control is provided by the carboxyl terminus of Tie2, which appears to obscure the substrate binding site (Fig. 1).

In the unphosphorylated state, the hydroxyl groups of two important tyrosine residues, Tyr1101 and Tyr1112 (murine residue numbers), are hydrogen-bonded to surrounding residues, which may stabilize the C tail in this inhibitory conformation. Glutamate 1120 also appears to stabilize this conformation by forming a salt bridge with the arginine residue at position 914. These novel inhibitory mechanisms suggest that Tie2 activation is distinct from that of other RTKs and that it may be more complex.

In this report, we investigated the role of the C tail in Tie2 kinase activation, downstream signaling, and function. To do this, we generated Tie2 kinase constructs in which C-terminal amino acid residues were deleted based on their apparent positions in the Tie2 crystal structure. Our results demonstrate that deletion of the C tail results in significant increases in receptor autophosphorylation and kinase activity. Moreover, these increases correlate with enhanced downstream signaling through Akt and inhibition of apoptosis. These findings may have implications for the design of small molecule Tie2 kinase inhibitors and for our understanding of vascular morphogenesis.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—NIH 3T3 cells were from American Type Culture Collection. Mouse monoclonal anti-phosphotyrosine antibodies were from Santa Cruz Biotechnology (clone PY20) and BD-Transduction Laboratories (RC20-AP) (Lexington, KY). Anti-phospho-Akt (Ser177), anti-phospho-p44/42-ERK (Thr202/Tyr204), and anti-cleaved caspase-3 (D175) rabbit polyclonal antibodies were purchased from Cell Signaling Technologies (Beverly, MA). Rat monoclonal anti-c-fms...
Generation of Recombinant Glutathione S-Transferase (GST)-Kinase Fusion Proteins—The recombinant wild type (WT) GST-Tie2 kinase fusion protein expressed in Sf9 insect cells has been described previously (13, 14). The Tie2 C tail deletion mutant (ΔCT) was generated by PCR using the wild type kinase domain cDNA as a template with the following primers, which delete amino acid residues 1108 to 1123 in the C tail of murine Tie2: forward primer, 5'-GACCGAATTCCTGGAGCT-TAAGAGACAAAATGTTCCAAAGG-3'; reverse primer, 5'-GACCGCGG-GCCGCTTGAATTACAGTGTGGTTCACGTATGT-3'. The resulting cDNA was sequenced and subcloned into the baculovirus expression vector pV1KS (15), which is derived from pVL-1393 (Pharmingen) and contains sequences for GST, a heart muscle kinase tag, and an influenza hemagglutinin epitope. Additional sequences result in a baculovirus-expressed GST-ΔCT protein that is larger than GST-Tie2-WT, despite the loss of 16 amino acid residues. The GST-ΔCT protein was expressed in Sf9 insect cells and purified as described previously (13).

Generation of Cell Lines Expressing Tie2 and ΔCT—NIH 3T3 cells expressing Tie2-WT have been described previously (14). To generate Tie2-ΔCT, the ΔCT cDNA replaced that of the WT Tie2 kinase in the retroviral expression plasmid LN CX-fTie2 (14), resulting in fusion of ΔCT with the extracellular and transmembrane domains of human c-fms. Recombinant ecotropic retroviruses were generated, and monoclonal cell lines were selected and analyzed as described previously (14).

In Vitro Kinase Assays—Tie2 kinase autophosphorylation was analyzed essentially as described previously (13). Briefly, ~500 ng of each fusion protein was purified on glutathione-Sepharose and then maximally autophosphorylated in vitro by incubation for 20 min at room temperature with 50 μM unlabeled ATP and 10 μCi of [γ-32P]ATP (6,000 Ci/mmol; Amersham Biosciences) in 100 μl of kinase buffer. Proteins were eluted by boiling into Laemmli sample buffer, separated by SDS-8% PAGE, transferred to nitrocellulose, and evaluated by autoradiography and by Western blotting with anti-phosphotyrosine.

Tie2 kinase activity was analyzed using the previously described D1–15 peptide (biotin-Ahx-LEARVAYEGWVAGKKK-NH2) (16) as a substrate. The peptide was chemically synthesized by BioSource International (Hopkinton, MA). Approximately 500 ng of each GST-kinase fusion protein was phosphorylated on glutathione-Sepharose in triplicate and then maximally autophosphorylated in vitro by incubation for 20 min at room temperature with 50 μM unlabeled ATP and 2 μM peptide in 100 μl of kinase buffer. Kinase reactions were carried out at room temperature for the indicated time and stopped by the addition of 50 μl EDTA. The Sepharose beads were pelleted by centrifugation, and the supernatant containing the biotinylated peptide substrate was bound to wells of a streptavidin-coated 96-well plate (StreptaWell; Bio-Rad) by incubation at room temperature for 1 h. After washing with phosphate-buffered saline, the wells were incubated with alkaline phosphatase-conjugated anti-phosphotyrosine (RC20-AP; 1:1000) for 1 h at room temperature. RC20-AP binding was detected by incubation with p-nitrophenyl phosphate (Roche Molecular Biochemicals catalog no. 726923) for 30 min at room temperature, which results in a reaction product detectable at 405 nm. Absorbance was measured on a Molecular Devices Vmax kinetic microplate reader.

Immunoprecipitation and Western Blotting—NIH 3T3 cells expressing chimeric Tie2 receptors were grown in 100-mm dishes until just subconfluent, serum-starved overnight in Dulbecco's modified Eagle's medium without serum, and then stimulated with CSF-1 (500 ng/ml) for 10 min at 37 °C. The cells were lysed, and the receptors were immunoprecipitated as described previously (14) except that immunoprecipitates were immobilized on goat anti-rat IgG-agarose (Sigma). Western blotting was performed with the indicated antibodies, as described previously (14, 17). To detect Tie2, a polyclonal antibody against the juxtamembrane domain was used, since Tie2-ΔCT lacks the epitope recognized by the previously described C tail antibody (13).

Apoptosis Assays—Apoptosis of Tie2-expressing cells was quantified following treatment with staurosporine (Calbiochem) using two different assays. To quantify caspase-3 activation, cells were plated at 1 × 106 cells/well of a six-well plate, grown for 24 h, and then serum-starved 16 h in the absence or presence of CSF-1 (500 ng/ml). Apoptosis was then induced by incubation with staurosporine (0.5 μM) for 2 h at 37 °C. The cells were lysed, and caspase-3 activity was analyzed using the colorimetric ApoAlert Caspase-3 Assay Kit (CLONTECH) according to the manufacturer's instructions (17). Alternatively, the cells were plated in wells of a 24-well plate and grown to confluence. The cells were again serum-starved in the absence or presence of CSF-1 (500 ng/ml), and apoptosis was induced with staurosporine, but apoptosis was quantified using the Cell Death Detection ELISA-Plus Kit (Roche Molecular Biochemicals catalog no. 1774425).

Statistical Analysis—All results were expressed as the mean ± S.E. Statistical analysis was performed using the one-tailed Student's t test (two-sample, unequal variance), and p < 0.05 was considered statistically significant.

RESULTS

Deletion of the Tie2 C Tail Enhances Autophosphorylation and Kinase Activity in Vitro—The crystal structure of the Tie2 kinase was recently solved (12) and suggested a potentially novel autoinhibitory role for the carboxyl terminus. In this structure, the initial residues of the Tie2 C tail (1101–1107; murine residue numbers) point away from the active site (Fig. 1). Immediately after tyrosine 1107, the C tail angles upward toward the active site, where it appears to obscure access to the substrate binding pocket of the kinase. This conformation appears to be maintained, in part, by hydrogen bonding of the hydroxyl residue of tyrosine 1112 and packing of its phenyl ring by adjacent residues. In addition, the C tail appears to be stabilized by salt bridging of glutamate 1120 to nearby residues. We investigated the role of the C tail in Tie2 autophosphorylation and kinase activation by generating a mutant in which the C-terminal 16 amino acid residues, including Tyr1112 and Glu1120 (residues 1108–1123), were deleted (termed ΔCT). Notably, this mutation preserves tyrosine 1101, which has been shown to regulate phosphatidylinositol (PI) 3-kinase/Akt signaling by Tie2 (14). GST-Tie2 kinase fusion proteins were generated and purified on glutathione-Sepharose, and autophosphorylation was evaluated by both Western blotting with anti-phosphotyrosine and incorporation of [γ-32P]ATP. Consistent with our previous results, Tie2-WT was detectably autophosphorylated in both assays (Fig. 2). Deletion of the C tail dramatically enhanced Tie2 autophosphorylation, despite the removal of Tyr1112, which was previously shown to be an important autophosphorylation site (13).

Based on this increase in autophosphorylation, we asked whether the kinase activity of the ΔCT mutant against an exogenous substrate would be enhanced compared to Tie2-WT. To do this, we performed in vitro kinase reactions using a synthetic peptide substrate that has been shown to be phos-
Deletion of the C tail enhances Tie2 autophosphorylation and downstream signaling in vivo—We showed previously that chimeras consisting of the extracellular and transmembrane domains of the CSF-1 receptor and the cytoplasmic kinase domain of Tie2 (designated fTie2) are autophosphorylated and signal through PI 3-kinase/Akt following CSF-1 stimulation (14). To determine whether the increased Tie2 autophosphorylation and kinase activity of the ΔCT mutant observed in vitro were biologically relevant, we generated stable cell lines expressing fTie2 receptors bearing the ΔCT mutation. Receptor expression and CSF-1-induced autophosphorylation were evaluated in three independent clones of fTie2-ΔCT and compared to that of fTie2-WT. Consistent with our previous findings, CSF-1 induced a modest but detectable increase in fTie2 tyrosine phosphorylation (Fig. 4A). In contrast, all three clones of ΔCT displayed markedly greater ligand-induced phosphorylation than fTie2-WT. Moreover, the ΔCT clones all demonstrated basal receptor phosphorylation that was similar to or greater than that of WT in the presence of CSF-1. One clone of ΔCT-expressing cells, ΔCT.9, had receptor expression levels that were similar to those of fTie2-WT. For this cell line, both basal and CSF-1-dependent autophosphorylation were dramatically increased compared with WT. These findings suggest that the ΔCT mutant is at least partly active in the absence of ligand, consistent with the release of an autoinhibitory mechanism.

We next asked whether the enhanced autophosphorylation of ΔCT corresponded with enhanced Tie2 signaling. Perhaps the best characterized signaling pathway for Tie2 is the PI 3-kinase/Akt pathway (14, 18–20). Tie2 has also been shown to induce ERK phosphorylation (21), although the activation of this signaling protein does not correlate with mitogenesis, and its precise role in Tie2 function is not yet known. Because receptor expression and autophosphorylation were nearly identical in two of the fTie2-ΔCT clones (clones 7 and 10), we chose one of these lines for subsequent analysis of signaling and cellular responses. Therefore, responses of fTie2-WT were compared to those of ΔCT.9, which had receptor expression comparable to that of WT, and compared to ΔCT.7 for analysis of a lower receptor-expressing clone. CSF-1 induced a modest but detectable phosphorylation of Akt and ERK-1 and -2 in fTie2-WT cells (Fig. 4B). For both clones of ΔCT tested, these proteins were basally phosphorylated as much or more than by fTie2-WT in the presence of ligand, and CSF-1 induced a further increase in their phosphorylation. These findings indicate that the enhanced autophosphorylation and kinase activity of ΔCT translate into increased recruitment and activation of downstream substrates, such as PI 3-kinase.

The C Tail Deletion Enhances Tie2-mediated Survival—Activation of the PI 3-kinase/Akt signaling pathway by Tie2 has been linked to inhibition of apoptosis (18–20); therefore, we investigated whether the enhanced signaling by ΔCT improved cell survival. Because the cell lines described here were derived from NIH 3T3 cells, we induced apoptosis by treatment with staurosporine in the absence of serum. Two different clones of ΔCT-expressing cells and fTie2-WT cells were serum-starved overnight in the absence or presence of CSF-1 and then treated with staurosporine. Cell lysates were analyzed by Western blotting for Akt phosphorylation and cleaved caspase-3, which correlates with the induction of apoptosis. Following overnight CSF-1 treatment, Akt was again phosphorylated in fTie2-WT cells, and this effect correlated with a reduction in caspase-3 cleavage (Fig. 5). In the low receptor-expressing cells, ΔCT.7, Akt phosphorylation was detectable at baseline and increased with CSF-1 treatment. Consistent with this finding, cleaved caspase-3 was only minimally detectable in the absence or presence of CSF-1. In the high receptor-expressing cells, ΔCT.9, Akt phosphorylation and caspase-3 cleavage were more dramatically altered compared with Tie2-WT, and virtually no cleaved caspase-3 was detectable in these cells.

To quantify the effects of ΔCT expression on apoptosis, we assayed caspase-3 activity and histone-associated DNA frag-
results, CSF-1 stimulation of fTie2-WT or -ΔCT were either left unstimulated or were stimulated with CSF-1 (500 ng/ml) and then lysed. Receptors were immunoprecipitated with an antibody against c-fms, resolved by SDS-8% PAGE, and Western blotted sequentially with antiphosphotyrosine and anti-Tie2 antibodies. B, cells were treated and lysed as described for A, and an aliquot of total cell lysate from each sample was resolved by SDS-8% PAGE and Western blotted sequentially with the indicated antibodies. Equal protein loading was confirmed by blotting with anti-α-tubulin.

FIG. 4. Deletion of the Tie2 C tail enhances ligand-induced autophosphorylation and downstream signaling. A, NIH 3T3 cells expressing fTie2-WT or three independent clones of ΔCT were grown 24 h in full growth medium and then serum-starved overnight in the absence or presence of CSF-1 (500 ng/ml). Apoptosis was induced by treatment with staurosporine, and cell lysates were used to analyze fTie2 expression, Akt phosphorylation, and caspase-3 cleavage by Western blotting. Equal protein loading was confirmed by blotting with anti-α-tubulin.

FIG. 5. Enhanced Akt phosphorylation by Tie2-ΔCT correlates with reduction in caspase-3 cleavage. NIH 3T3 cells expressing fTie2-WT or two independent clones of ΔCT were grown 24 h in full growth medium and then serum-starved overnight in the absence or presence of CSF-1 (500 ng/ml). Apoptosis was induced by treatment with staurosporine, and cell lysates were used to analyze fTie2 expression, Akt phosphorylation, and caspase-3 cleavage by Western blotting. Equal protein loading was confirmed by blotting with anti-α-tubulin.

A variety of different regulatory mechanisms have been demonstrated for RTKs, and many of these have been either identified or confirmed by crystal structure analyses (23). For example, tyrosine 1162 in the activation loop of the insulin receptor is bound in cis in the active site to prevent access by both ATP and peptide substrates (24); the activation loop of the fibroblast growth factor receptor-1 (FGFR1) and vascular endothelial growth factor receptor-2 kinases are positioned to block substrate binding (25, 26); and the juxtamembrane domain of EphB2 interferes with both ATP positioning within the catalytic site and proper positioning of the activation loop for catalysis (27). Of the published RTK crystal structures, only that of Tie2 suggests negative regulation by its C terminus. However, many RTKs that have been crystallized lack certain domains, such as the C tail, which limits the conclusions that can be drawn about their precise mechanisms of regulation. Of particular relevance here, the FGFR1 protein used to analyze its crystal structure lacked the C tail (25). Interestingly, Lorenzi et al. (28) later demonstrated that several C-terminal deletion mutations of FGFR2 resulted in increased transforming activity of the receptor, although no increases in basal tyrosine phosphorylation or ERK activation were observed. In fact, several of these deletion mutants displayed reduced or absent autophosphorylation in response to fibroblast growth factor, probably due to the removal of key autophosphorylation sites, but the corresponding tyrosine to phenylalanine mutants displayed similar transforming activity. These findings suggest that negative regulation by the FGFR2 C tail is a result of specific signaling pathways regulated by this domain rather than a structural phenomenon.

A negative regulatory role for the C tail has been demonstrated biochemically for at least two other RTKs, although in neither case has the kinase domain been crystallized. Substitution of the C-terminal 50 amino acid residues of c-fms with 11 residues of v-fms results in enhanced receptor autophosphorylation and transforming activity despite the removal of one tyrosine residue, although it is not clear whether this residue is autophosphorylated in vivo (29). Akiyama et al. (30) demonstrated that deletion of the C-terminal 230 residues of ErbB2 enhanced the receptor’s kinase activity as well as its cellular transforming ability. The C tail of ErbB2 contains ~270 residues, which is much longer than that of Tie2 or most other RTKs. As a result, perhaps it is not surprising that it could interfere with enzymatic activity, although structural data are not available to confirm the mechanism of this inhibition. By comparison, the ΔCT mutation used for our studies removes a relatively short segment of only 16 residues, and the dramatic effects of this mutation are consistent with structural data.
indicating that the C tail inhibits substrate access by binding to nearby residues.

Together, structural and biochemical studies of RTKs have provided insights into the mechanisms of kinase activation following ligand-mediated oligomerization (31). Typically, activation requires autophosphorylation of tyrosine residues within the activation loop, resulting in an “open,” active conformation that allows access to ATP and peptide substrates. The A-loop of Tie2 is in a more “active-like” conformation that resembles that of the active, autophosphorylated insulin receptor kinase (12). Deletion of the C tail of Tie2 in vivo resulted in high basal levels of autophosphorylation that were enhanced further by ligand. This basal autophosphorylation could not be attributed to high receptor expression, since fTie2-WT cells with equal or greater levels of expression showed less basal phosphorylation. These findings suggest that an early step in Tie2 activation is a conformational change that displaces the C tail from its autoinhibitory position. Although it is not clear whether full activation of Tie2 requires phosphorylation of Tyr992 in the A-loop or a conformational change of the nucleotide binding loop, release of autoinhibition by the C tail would probably allow such changes to proceed more efficiently. This notion is supported by our in vitro results. In the kinase activity assay, a delay in the activation of both wild type and ΔCT was observed, after which activity of ΔCT increased significantly compared with that of wild type. In this experiment, the kinases were not subjected to an autophosphorylation reaction prior to the addition of substrate peptide, so it is possible that important tyrosine residues like Tyr992 had not yet been phosphorylated. Therefore, the delay in kinase activation might represent the requirement for A-loop tyrosine phosphorylation, and we predict that removal of the C tail would facilitate this process. Taken together, our results indicate that the Tie2 C tail provides an important level of negative regulation of Tie2 kinase activity.

It has been suggested that hydrogen bonding of the hydroxyl group of Tyr1112 might maintain the C tail in its inhibitory conformation and that autophosphorylation of this residue would release the inhibition (12, 23). Our studies partially support this hypothesis, since the ΔCT mutant lacks Tyr1112. However, it is impossible to determine from our studies whether phosphorylation of Tyr1112 would be sufficient to alter the conformation of the C tail. Importantly, the removal of Tyr1112 by the ΔCT mutation enhanced autophosphorylation, kinase activity, and downstream signaling by Tie2. In contrast, a Y1112F mutation decreases Tie2 autophosphorylation (13) and kinase activity2 in vitro. The different effects of these two mutations on receptor phosphorylation and activity suggest that the hydroxyl residue of Tyr1112 does not play a structural role in autoinhibition by the Tie2 C tail. However, like the FGFR2 mutations described above (28), it is possible that Tyr1112 could play a negative regulatory role through specific signaling partners that bind this residue. For example, Tyr1112 was shown to be the site of association of the protein-tyrosine phosphatase Shp2 (13), which has been shown to dephosphorylate and negatively regulate the platelet-derived growth factor β receptor (32). Studies are currently under way to elucidate which residues are responsible for regulating autoinhibition by the Tie2 C tail.

Recently, there has been great interest in understanding the mechanisms of action of Tie2 for therapeutic purposes. Disruption of Tie2 signaling with a soluble receptor protein results in significant inhibition of tumor angiogenesis, growth, and metastasis (5–7). These studies indicate that Tie2 is a potentially important target for the prevention of tumor angiogenesis. As a result, small molecule inhibitors of Tie2 are actively being designed and tested for the treatment of cancer. By shedding light on the mechanisms of Tie2 activation, our results may aid in the development of such inhibitors, which could potentially be designed based on residues in the C tail that inhibit substrate peptide access.

The current results may also provide insights into the mechanisms of vascular morphogenesis. Two different inherited Tie2 mutations have been identified and linked to vascular malformations (33, 34). Both of these mutations enhance Tie2 autophosphorylation, although their effects on Tie2 kinase activity have yet to be determined. In both cases, the mutations result in vascular dysmorphogenesis characterized by abnormal contacts between endothelial cells and smooth muscle cells or pericytes. One of these mutations, R897W, has been shown to enhance downstream activation of Stat1 (35). Otherwise, little is known about the signaling pathways and downstream cellular responses that are affected by these Tie2 mutations. Our results with the ΔCT mutant suggest that hyperactivating mutations of Tie2 result in dysregulated, enhanced activation of its normal signaling pathways, such as PI 3-kinase/Akt, which enhances cell survival. In this manner, vascular malformations caused by the Tie2 mutations may behave like cancer (23). Endothelial cells expressing mutant Tie2 receptors may be resistant to apoptosis despite damage that would normally lead to their clearance from the vasculature (e.g., during vascular remodeling following wounding). Further studies will be necessary to determine whether the activating Tie2 mutations result in aberrant signaling compared with wild type Tie2. Nonetheless, continued understanding of the mechanisms of Tie2 activation is likely to facilitate progress in the treatment of numerous vascular diseases.

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2 X.-L. Niu and C. D. Kontos, unpublished observations.
