FUNCTIONS OF THE POINTED DOMAIN
WITHIN THE ETS GENE FAMILY

by

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This dissertation has been read by each member of the following supervisory committee and by majority vote has been found to be satisfactory.
To the Graduate Council of the University of Utah:

I have read the dissertation of John Seidel in its final form and have found that (1) its format, citations, and bibliographic style are consistent and acceptable; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the supervisory committee and is ready for submission to The Graduate School.

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ABSTRACT

All members of the ets family of transcription factors conserve the DNA binding ETS domain that recognizes the core sequence 5'-GGA(A/T)-3'. Despite conserving this domain, individual ets proteins can perform specific functions. Many unique features exist among ets proteins that establish this diversity. One feature, the Pointed (PNT) domain, is a region of 80 amino acids conserved among only a subset of ets proteins. The function of the PNT domain is not clear, but it is proposed to mediate homotypic and heterotypic protein-protein interactions.

This thesis investigates the function of the PNT domain of four ets proteins. Surprisingly, this domain was found to serve different functions within the family. First, PNT domains can have different oligomerization states. The Ets-1 PNT domain, composed of five alpha helices that assume a globular fold, is monomeric in solution. In contrast, the PNT domain of the ets family member TEL formed multimers under a variety of in vitro conditions. Second, PNT domains have different protein partners. In an affinity chromatography experiment, a fragment of Ets-1 spanning the PNT domain and MAPK site bound the mitogen-activated protein kinases (MAPKs) ERK1 and/or ERK2. These kinases phosphorylate Ets-1 and Ets-2 at a MAPK site N-terminal to the PNT domain. Kinase assays identified a docking site on the surface of the PNT domain of Ets-1 important for interacting with ERK2 and Ras/MAPK signaling. The docking site is conserved in sequence and in function in the PNT domain of Ets-2 but not in the
PNT domain of the ets family member GABPα. The docking site sequence is also not well-conserved in other PNT domains. These results highlight the PNT domain as a conserved structural element whose distinct surface features generate specificity of function among ets proteins.
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CHAPTER 1

INTRODUCTION
Differential gene expression and regulatory transcription factors

Most cells within an organism contain identical genetic information. Through differential gene expression, use of this information is temporally and spatially regulated. The ability to differentially express genes is a fundamental process critical to the proper maintenance of an organism. Differential gene expression allows an organism to develop multiple cell-types, each with unique functions and diverse properties. In addition, the ability to differentially express genes allows cells to respond to environmental stimuli. Understanding the molecular mechanisms that underlie differential gene expression is vital to many areas of basic and applied biology, including embryology, oncogenesis, and stem cell research.

One group of proteins required for differential gene expression is regulatory transcription factors. Regulatory transcription factors are sequence specific DNA binding proteins that bind enhancer elements 5' of genes and modulate transcriptional initiation. These proteins can perform different activities that affect transcription (Fig. 1.1). First, these proteins can serve architectural roles by altering the structure of the DNA composing the enhancer of a gene. Second, they can recruit other regulatory transcription factors to the enhancers of specific genes. Third, transcription factors can recruit the machinery required for alteration of chromatin structure, such as corepressors and coactivators. Last, transcription factors can recruit the basal transcriptional machinery, including the TFIID complex, RNA polymerase II, and the Mediator complex. These activities determine which genes are transcriptionally on or off.
Figure 1.1 Roles of regulatory transcription factors. a) Alter DNA structure (architectural protein); b) recruit other regulatory transcription factors to an enhancer; c) recruit chromatin-modifying complexes to nearby nucleosomes; d) recruit the basal transcriptional machinery to the promoter. Other roles are possible.
Specificity of function among a gene family

Multiple regulatory transcription factors exist in an organism, and many show sequence similarity within their DNA binding domains. This similarity can be used to group transcription factors into gene families (Harrison, 1991; Pabo and Sauer, 1992). For instance, the leucine zipper/basic region (bZIP), MADs-box, Runt domain, and HMG-box define different transcription factor families. Because of a shared DNA binding domain, family members often bind similar DNA sequences.

Despite conserving the DNA binding domain, all family members do not perform identical functions. Genetic studies suggest that individual members of a family can regulate different genes. Different functions arise, even for family members that are co-expressed in the same cell. These observations raise the question of how specificity of function is generated among a gene family.

Specificity of transcription factor function can be considered at two levels. The first level is target specificity: which genes do individual members of a transcription factor family regulate? This question is technically challenging to address, due in part to co-expression of multiple family members. The second level is molecular specificity: what features define distinct functions among individual family members? For example, why are some members of a family activators of transcription whereas others are repressors? This molecular concept of specificity is more tractable to investigate than target specificity and can yield greater insight into family member function.
The \textit{ets} family of regulatory transcription factors

One family of regulatory transcription factors found in all metazoans is the \textit{ets} family of proteins (Graves and Petersen, 1998) (Fig. 1.2). \textit{Ets} genes number 8 in \textit{Drosophila}, 10 in \textit{C. elegans}, and 25 in humans. All \textit{ets} proteins conserve an 85 amino acid DNA binding ETS domain that folds into a winged-helix-turn-helix structure (Liang et al., 1994; Donaldson et al., 1996; Pio et al., 1996; Werner et al., 1997; Batchelor et al., 1998; Mo et al., 1998; Mo et al., 2000). Biochemical and structural studies of a subset of \textit{ets} family members demonstrate that the ETS domain binds the core DNA sequence 5'-GGA(A/T)-3' (Graves and Petersen, 1998). It is proposed that all \textit{ets} family members bind enhancer elements containing a core GGA sequence.

\textit{Ets} proteins can perform a variety of functions, despite conserving a highly similar DNA binding domain. This has been demonstrated by gene disruption studies in model organisms. In mice, for instance, seven \textit{ets} genes have been targeted for disruption, including \textit{ETS-1}, \textit{ETS-2}, \textit{PU.1}, \textit{TEL}, \textit{FLI-1}, \textit{SPI-B}, and \textit{ER81} (Bartel et al., 2000), and distinct phenotypes were observed for mutation of each gene. For example, targeting of \textit{TEL} resulted in defects in bone marrow hematopoiesis (Wang et al., 1998), whereas targeting of \textit{ETS-1} resulted in defects in T cell and NK cell development (Barton et al., 1998). Notably, these distinct phenotypes arose despite the fact that \textit{ETS-1} and \textit{TEL} are co-expressed in some of the same hematopoietic cells. Genetic studies in \textit{Drosophila} also point to unique functions for co-expressed \textit{ets} proteins. For example, both \textit{yan} and \textit{pnt} are expressed in the \textit{Drosophila} eye and are required for normal R7 photoreceptor development. However, \textit{yan} is a negative regulator of eye
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**Figure 1.2** Human members of the *ets* gene family. Family members are subgrouped based on sequence similarity within their DNA binding domains. Positions of the Pointed domain and ETS domain are noted.
development (Lai and Rubin, 1992; O'Neill et al., 1994; Rebay and Rubin, 1995) whereas \textit{pnt} is a positive regulator (Brunner et al., 1994; O'Neill et al., 1994).

It is apparent from these and other genetic studies that members of the \textit{ets} gene family display specificity with respect to the genes they regulate and the molecular mechanisms by which they function. This family can therefore be used to investigate the molecular features that define specificity among a gene family.

\textbf{Determinants of specificity of \textit{ets} function}

Many studies of \textit{ets} family members have focused on the similarities and differences in the regulation of DNA binding by the ETS domain. These studies have revealed subtle differences in the preferences for DNA binding sequences outside the 5'-GGA-3' core (Graves and Petersen, 1998; Szymczyna and Arrowsmith, 2000). Mechanisms have also been identified that modulate the affinity of ETS domain binding. For example, Ets-1 DNA binding activity is regulated by phosphorylation (Rabault and Ghysdael, 1994; Cowley and Graves, 2000), autoinhibition (Graves et al., 1998), and cooperative interactions with other DNA binding proteins (Goetz et al., 2000; Gu et al., 2000). These features show restrictive conservation and could contribute to the unique functions of the Ets-1 protein.

The work described in this thesis addresses the question of specificity by examining the function of two features conserved in a limited number of \textit{ets} proteins. The first is the Pointed (PNT) domain, a region of 80 amino acids conserved among approximately 40% of \textit{ets} proteins (Fig. 1.2). The function of the PNT domain is not well-established, but it is proposed to mediate protein-protein interactions. The second emphasis of study is the regulation of mitogen-activated protein kinase (MAPK)
phosphorylation of the ets proteins Ets-1, Ets-2, and Pnt-P2. Phosphorylation is
important for the transactivation function of these ets proteins, but how phosphorylation
is regulated is not well-understood.

Pointed domain

The PNT domain was originally defined by sequence homology that was first
recognized upon discovery of the Drosophila ets family member Pointed-P2 (Pnt-P2)
(Klambt, 1993). To date, the PNT domain has been found in 4/10 ets proteins
expressed in Drosophila, 2/10 C. elegans ets proteins, and 11/26 human ets proteins
(Fig. 1.2). A PNT domain is also present in the Drosophila protein Mae, the only
known PNT domain containing protein that lacks an ETS domain. The overall sequence
identity among PNT domains ranges between 15-65% (Fig. 1.3).

The PNT domains of ets family members also share weak sequence similarity to
the sterile-α-motif (SAM) domain. SAM domains are found in a diverse array of
proteins, including EPH-related receptor tyrosine kinases, yeast mating pheromone
response pathway proteins, Drosophila and vertebrate polycomb group proteins, and the
p53 related transcription factor p73 (Schultz et al., 1997). A closer relationship between
these domains is established by structural studies that demonstrate similarities in the
three dimensional structures among two PNT domains and three SAM domains
(Chapter 2; Slupsky et al., 1998; Chi et al., 1999; Smalla et al., 1999; Stapleton et al.,
1999; Thanos et al., 1999a; Thanos et al., 1999b; Kim et al., 2001; Wang et al., 2001).
These similarities suggest that these domains are derived from a common ancestor
(Shao and Grishin, 2000).
Figure 1.3 Sequence alignment of the PNT domain. Human, (H); mouse, (M); C. elegans, (C); and Drosophila melanogaster (D). Drosophila Mae does not contain an ETS domain. Residues conserved in greater than 50% of proteins are shaded in black; residues with conservative substitutions in greater than 50% of proteins are shaded in gray. The positions of α-helices (boxes) of the Ets-1 PNT domain are shown (Slupsky et al., 1998). Helix H1 is not well-conserved and may not assume a helical structure in all PNT domains. Helices H2-H5 are conserved in TEL (Kim et al., 2001).
Proposed functions of the PNT domain among ets family members

Multiple activities have been suggested for the PNT domain of the ets family member TEL. One activity, self-association, was originally proposed upon identification of a fusion protein in which the PNT domain of TEL was linked to the catalytic domain of the platelet derived growth factor receptor β (PDGFRβ) as a result of a chromosomal translocation (Golub et al., 1994). The PNT domain replaced a ligand binding domain in PDGFRβ that is responsible for its oligomerization. Subsequent structural studies demonstrated that the PNT domain of TEL can form a helical polymer (Kim et al., 2001). Polymerization is proposed to be responsible for multimerizing the fused tyrosine kinases and leading to oncogenic signaling (Jousset et al., 1997). Polymerization may also be important for the role of TEL as a transcriptional repressor (Lopez et al., 1999).

Tentative evidence indicates that the PNT domain of TEL mediates interactions with PNT domains of two other ets family members. First, two-hybrid data suggest that a PNT domain containing fragment of TEL interacts with a fragment of Fli-1 containing its PNT domain (Kwiatkowski et al., 1998). This interaction was proposed to repress the transactivation activity of Fli-1 (Kwiatkowski et al., 1998). Second, the PNT domain of TEL is also required for the ability of TEL to interact with highly related protein TEL2 (Potter et al., 2000), but a function for this interaction was not tested. However, in neither case was it demonstrated that the PNT domain of either protein directly mediated the interactions.

The TEL PNT domain is also reported to interact with two non-ets proteins. One partner is UBC9, an enzyme that conjugates a small, ubiquitin-like protein termed
SUMO (Chakrabarti et al., 1999; Chakrabarti et al., 2000). Studies with TEL suggest that its PNT domain is modified by UBC9 and that this modification results in alteration of the nuclear localization of TEL (Chakrabarti et al., 2000). The second partner is mSin3A, a component of a histone deacetylase complex that represses transcription (Fenrick et al., 1999; Wang and Hiebert, 2001). This interaction is thought to be responsible for the activity of TEL as a transcriptional repressor. The interaction with mSin3A is a matter of controversy, however, as other mSin3A binding sites in TEL have been mapped.

The PNT domains of Ets-1 and Ets-2 have been studied, but no functions have been firmly established. First, the PNT domain enhances transactivation mediated by sequences that lie between the PNT domain and ETS domain in Ets-1 (Schneikert et al., 1992). However, the PNT domains of Ets-1 and Ets-2 by themselves do not strongly activate transcription when fused to a heterologous DNA binding domain (Schneikert et al., 1992). Second, a two-hybrid interaction action assay suggested an interaction of UBC9 with a PNT domain containing fragment of Ets-1. However, this interaction was not localized to the PNT domain (Hahn et al., 1997). Third, a fragment of Ets-1 containing the PNT domain binds the corepressor Daxx (Li et al., 2000). However, like the UBC9 interaction, it was not demonstrated whether the Ets-1 PNT domain is necessary and sufficient for the interaction with Daxx. Last, unlike the TEL PNT domain, which can exist as a polymer, the Ets-1 PNT domain does not self-associate (Chapter 2; Slupsky et al., 1998). Thus, although partners are proposed, the Ets-1 PNT domain has no established function.
The PNT domain has been studied in three *Drosophila ets* proteins. Interaction of the PNT domain of Pnt-P2 and Yan with the PNT domain of Mae appears to enhance the phosphorylation of Pnt-P2 and Yan outside their PNT domains (Baker et al., 2001). The mechanism of this enhancement is not known. An apparent ortholog of Mae in vertebrates has not been described, so it is not known if this function is conserved in higher organisms.

In summary, although several protein partners for the PNT domains of various ets family members have been proposed (Table 1.1), the function of the domain is not well-established. The data most strongly support a role for the TEL PNT domain in oligomerization, but this function does not appear to be conserved among other PNT domains. Due to differences in oligomerization states and function in transactivation assays of a subset of PNT domains, it appears that PNT domains may perform different roles in different ets proteins. Indeed, a common interaction partner for all PNT domains has not been described, and no common function for all PNT domains has been proposed. This indicates that investigation of different PNT domains will lend insights into novel and distinct functions for ets proteins.

**Phosphorylation of ets proteins**

Phosphorylation is another method of modulating the specific functions of ets proteins. Many, if not all, ets proteins are regulated by phosphorylation. Known ets phosphoproteins include vertebrate Ets-1 (Koizumi et al., 1990), Ets-2 (Fujiwara et al., 1988), TEL (Poirel et al., 1997), GABPα (Flory et al., 1996; Fromm and Burden, 2001), ERG (Murakami et al., 1993), Elk-1 (Gille et al., 1995), SAP-1 (Strahl et al., 1996), SAP-2 (Maira et al., 1996), ER81 (Janknecht, 1996a), ERM (Janknecht et al., 1996b),
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Spi-B (Mao et al., 1996), PU.1 (Pongubala et al., 1993), ERF (Sgouras et al., 1995),
ESE-3 (Tugores et al., 2001) the Drosophila proteins Yan and Pnt-P2 (Brunner et al.,
1994; O'Neill et al., 1994), and the C. elegans protein LIN-1 (Tan et al., 1998).

Phosphorylation can affect the activities of ets proteins by a variety of
mechanisms. For instance, phosphorylation of Elk-1 enhances its DNA binding activity
and transcriptional regulation (Yang et al., 1999). Phosphorylation of Yan is
responsible for its nuclear export and degradation (Rebay and Rubin, 1995). PU.1
phosphorylation affects its protein partnership with PIP (Eisenbeis et al., 1995).
Phosphorylation of Ets-1 near its ETS domain represses DNA binding activity (Rabault
and Ghysdael, 1994; Cowley and Graves, 2000). Thus, phosphorylation can establish
unique functions among members of the ets gene family.

The work described in this thesis investigates the phosphorylation of Ets-1 and
Ets-2 by mitogen-activated protein kinases (MAPKs). These vertebrate ets proteins and
Drosophila Pnt-P2 are phosphorylated at a MAPK site 16 amino acids N-terminal of
their PNT domain (Fig. 1.4). Phosphorylation of this site results in an upregulation of
the ability of these proteins to serve as transcriptional activators (Brunner et al., 1994;
O'Neill et al., 1994; Yang et al., 1996). Physiologically, MAPK phosphorylation of the
conserved site in Pnt-P2 is required for R7 photoreceptor development in the
Drosophila eye (Brunner et al., 1994; O'Neill et al., 1994). The mechanism by which
phosphorylation enhances transactivation is not known.

Several kinases have been identified that phosphorylate Ets-1, Ets-2, and Pnt-P2.
Most evidence indicates that these kinases are the MAPKs ERK1 and/or ERK2
(ERK1/2) in vertebrates and the highly related MAPK termed Rolled in Drosophila.
Figure 1.4 Schematic of Ets-1, Ets-2, and Pnt-P2. The MAPK phosphorylation site is denoted by an asterisk. The sequence of the MAPK site is shown below the schematic. The underlined residue is the phosphoacceptor.
There is also evidence that Ets-2 can be phosphorylated by the MAPK jun-N-terminal kinase (JNK) (Smith et al., 2000). However, there are no reports of phosphorylation of this site by other MAPKs. This suggests that determinants may exist among MAPKs that direct the specificity of phosphorylation of these ets proteins. The work described in this thesis identifies a potential role for the PNT domain of Ets-1, Ets-2, and Pnt-P2 in directing the specificity of phosphorylation. The information below provides perspective into how MAPK signaling pathways are regulated and how MAPKs recognize specific substrates.

**MAPK signaling pathways**

MAPK signaling pathways are critical modulators of many biological processes, including differentiation, proliferation, inflammation, and apoptosis (Schaeffer and Weber, 1999). MAPK pathways are activated by diverse extracellular stimuli, including growth factors and inflammatory cytokines. The extracellular signals ultimately activate a three-kinase module, a defining feature of MAPK signaling pathways. This module is composed of a MAPK kinase kinase (MAPKKK) or MEKK, a MAPK kinase (M KK) or MEK, and a MAPK (Fig 1.5).

There are several distinct MAPK modules, each of which is primarily responsible for responding to unique types of extracellular stimuli. For instance, the ERK1/2 module usually responds to growth factors, whereas the JNK and p38 modules respond to cellular stresses. The fidelity of the original signal is maintained due to minimal cross-talk among the components of the distinct three-kinase modules. For example, MEK1 and MEK2 only activate ERK1 and ERK2, and M KK6 and M KK3
Stimulus Growth factors Inflammatory cytokines cellular stress

MAPKKK Raf MEKK MLK 

MAPKK MEK1/2 MKK4/7 MKK3/6 

MAPK ERK1/2 JNK p38 MAPK

Response Proliferation, Differentiation, Development Inflammation, Apoptosis, Development

**Figure 1.5** MAPK modules in mammalian cells. General MAPK pathway is shown on the left. MAPKKK (mitogen-activated protein kinase kinase kinase), MAPKK (MAPK kinase). Adapted from Schaeffer and Weber (1999).
only activate p38 MAPKs. Therefore, MAPKs can be activated to phosphorylate targets in response to distinct signals, resulting in a defined biological response.

**MAPKs**

Structural studies have defined the mechanism of activation of MAPKs by MAPKKs. MAPKs contain an N- and C-terminal lobe (Zhang et al., 1994), and the catalytic site lies in a cleft between the two lobes. As translated, MAPKs have very low catalytic activity. MAPKKs activate MAPKs through phosphorylation of two sites on an activation loop near the catalytic site. Phosphorylation of this loop results in several conformational changes near the catalytic site that greatly enhance the enzymatic activity of the MAPK (Canagarajah et al., 1997).

MAPKs, as well as cyclin-dependent kinases (CDKs), belong to a group of kinases termed proline-directed kinases (Clark-Lewis et al., 1991; Gonzalez et al., 1991). These kinases phosphorylate serine and threonine residues immediately N-terminal of a proline. In addition to this simple sequence, individual kinases can show subtle preferences for substrates based on sequences surrounding the phosphoacceptor. For instance, ERK1 and ERK2, but not other MAPKs, preferentially phosphorylate -(S/T)P- sites two residues C-terminal of a proline (-PX(S/T)P-) (Gonzalez et al., 1991). Despite these observations, it is not clear as to what determines which -(S/T)P- sequences in a protein will serve as a phosphoacceptor for MAPKs. In addition, it is also not clear what determines which MAPK will phosphorylate a given phosphoacceptor. Such differentiations are essential for generation of an appropriate biological response to an activation signal.
MAPK docking sites in transcription factors

One means by which MAPK substrate specificity is enhanced is through the activity of docking sites (Fig. 1.6). Docking sites are short sequence motifs within substrates that lie distal to the phosphoaccepter site (Holland and Cooper, 1999; Sharrocks et al., 2000). Several different docking motifs have been identified; for instance, in transcription factors two motifs defined by an LXL sequence or FXF sequence have been described. The LXL sequence lies N-terminal to phosphoacceptors; the FXF sequence lies C-terminal. Besides transcription factors, MAPK docking sites exist in a variety of proteins, including upstream and downstream kinases, phosphatases, and scaffold proteins.

A primary function of docking sites is to enhance the efficiency of phosphorylation of a substrate. This is most clearly demonstrated by enzymatic studies of docking sites in transcription factors. Substrates with site-directed mutations in LXL or FXF docking motifs have higher $K_m$ values in kinetic assays (Jacobs et al., 1999; Fantz et al., 2001). This increase can be interpreted as weaker binding affinity of the substrate for the kinase.

Docking sites can also differentially target MAPKs to substrates. For example, a docking site is required for ERK2 and JNKs to efficiently phosphorylate Elk-1 (Yang et al., 1998a). However, the docking site is not required for efficient phosphorylation by the p38 MAPKs. Thus, MAPKs appear to have different requirements for their ability to efficiently phosphorylate a substrate.

Docking sites affect the accuracy of phosphorylation. For example, an LXL containing docking motif is required for efficient phosphorylation of Jun at serine 63.
Figure 1.6 Model of MAPK docking site. Docking sites in substrates are thought to interact with MAPKs distal to the catalytic site. They can affect the efficiency, specificity, and accuracy of phosphorylation.
and serine 73 (Kallunki et al., 1996). However, mutating the docking site resulted in phosphorylation of other residues in c-Jun. In addition, the FXF sequence of Elk-1 directs ERK2 to phosphorylate serine 383, but the LXL motif directs the kinase to other sites (Fantz et al., 2001). Thus, docking sites can contribute to the selection of phosphoacceptor sites.

Although docking motifs appear to play a role in specifying the MAPKs that phosphorylate a substrate, the exact determinants of specificity are not fully defined. For instance, LXL motifs act as a docking site in several diverse proteins, including the ets proteins Elk-1 and SAP-1, the bZIP protein c-Jun, and the MAPKK MEK1. However, these motifs appear to target different MAPKs. For example, the LXL motif of SAP-1 enhances phosphorylation by the MAPKs p38β2, p38α, and ERK2 (Galanis et al., 2001), whereas the LXL motif of c-Jun targets only JNKs (Kallunki et al., 1996), and the LXL motif of Elk-1 targets both ERK2 and JNK (Jacobs et al., 1999). Therefore, other residues outside the LXL motif must generate the specificity of MAPK interaction.

The docking site defined by the FXF motif also does not appear to be sufficient to differentiate among MAPKs. This site is found C-terminal to a stretch of phosphoacceptors in the ets proteins LIN-1, Elk-1, and SAP-1 (Jacobs et al., 1998). In LIN-1 and Elk-1, the FXF sequence interacts with ERK2 (Jacobs et al., 1999). However, in SAP-1, the FXF sequence serves as an interaction site for both ERK2 and p38α (Galanis et al., 2001). As with the LXL motif, sequences outside the FXF sequence must play a role in defining the specificity of MAPK interactions.
Structural features of MAPK docking sites

Of the discovered MAPK docking sites, high-resolution structural information is available for only the binding site in the phosphatase MKP-3. The ERK2 binding domain of MKP-3 has an LXL motif preceded by basic residues. In the NMR solution structure, the KXXXLXL motif lies in a β-strand and the loop between the β-strand and a helix (Farooq et al., 2001). It is not clear if LXL motifs form a similar structure in other docking sites.

Interaction surfaces in MAPKs

Several biochemical studies indicate that docking sites can interact on different surfaces of MAPKs (Fig. 1.7). First, the bZIP protein c-Jun was proposed to interact with a helix adjacent to the catalytic site of JNK (Kallunki et al., 1994). Second, MEKs and phosphatases were reported to interact with two aspartic acids (D316 and D319) on ERK2 termed the Common Domain, or CD domain (Tanoue et al., 2000). Third, MAPK-activated protein kinases (MAPKAPKs) interact with p38 MAPK on nearby glutamic and aspartic acids termed the ED site (Tanoue et al., 2001). Fourth, hydrophobic residues in the MEK1 and Elk-1 docking site appear to interact with two tyrosines in ERK2 close to the CD domain and ED site (Xu et al., 2001). Unfortunately, there are no co-crystal structures of MAPKs with peptide substrates or substrate docking sites to provide a high-resolution model of these interactions.

MAPK docking conclusions

MAPK docking sites in substrates are critical regulators of the efficiency, specificity, and accuracy of phosphorylation. Given the multiple examples, it is
Figure 1.7 Docking site interaction surfaces on MAPKs. Two views of ERK2 are shown in spacefilling rendering, but all MAPKs have a similar structure. 1) Catalytic site; 2) interaction region for c-Jun on JNK; 3) CD domain for interaction with MAPKKs and phosphatases on ERK2; 4) ED site for interaction of MAPKAPKs on p38; 5) YY site for interaction with MEK1 and Elk-1 on ERK2.
possible that all proteins that are phosphorylated by MAPKs contain a docking site. Site-directed mutagenesis studies have not fully defined sequences that contribute to a docking site, though. Detailed structural information for docking sites would provide a better understanding of docking site function.

**Summary and aims**

The *ets* family of transcription factors is an excellent system to investigate the specific functions within a gene family. Beyond the ETS domain, many of the molecular features that contribute to the specificity of *ets* family function remain uncharacterized. At the commencement of my thesis work, the function of the PNT domain among *ets* family members was not known. Also poorly understood was the regulation of MAPK phosphorylation of Ets-1 and Ets-2.

The research described in this dissertation was directed at determining the structure and function of the PNT domain of TEL and Ets-1 and the MAPK site in Ets-1 and Ets-2. Chapter 2 of this dissertation describes the determination of the structure of the Ets-1 PNT domain and adjoining MAPK phosphorylation site, work performed primarily by Lawrence McIntosh's laboratory at the University of British Columbia. I contributed to the biochemical characterization of the fragment used for structural determination. Chapter 3 describes biochemical studies of the oligomerization state of the TEL PNT domain. The TEL PNT domain multimerizes, a phenomenon explained in subsequent reports as helical polymerization (Kim et al., 2001). Chapter 4 describes an affinity chromatography experiment in which a fragment of Ets-1 spanning the MAPK site and PNT was used to capture protein partners from a calf thymus nuclear extract. Over a dozen partners were determined to bind to the column. One was
identified as the protein UBC9 and another as the MAPKs ERK1 and/or ERK2.

Chapter 5 describes the investigation of the interaction of ERK2 with Ets-1. A novel ERK2 docking site was found in the PNT domain of Ets-1 that enhances phosphorylation of the N-terminal MAPK site. The docking site is conserved in Ets-2 but is not well-conserved among other PNT domains. The docking site is critical for Ras pathway mediated enhancement of the transactivation activities of Ets-1 and Ets-2. Finally, Chapter 6 provides a summary of these findings and a discussion of the functional and evolutionary significance of the PNT domain. In addition, future experiments are proposed that are designed to provide further insight into the function of the Ets-1 PNT domain and the mechanisms of MAPK docking site function.

References


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CHAPTER 2

STRUCTURE OF THE ETS-1 POINTED DOMAIN AND
MITOGEN-ACTIVATED PROTEIN KINASE
PHOSPHORYLATION SITE

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The experiments described in this chapter were performed in collaboration with Lawrence McIntosh’s laboratory at the University of British Columbia. My contribution was the partial trypsin proteolysis studies of Ets-1(29-138).
ABSTRACT  The Pointed (PNT) domain and an adjacent mitogen-activated protein (MAP) kinase phosphorylation site are defined by sequence conservation among a subset of ets transcription factors and are implicated in two regulatory strategies, protein interactions and posttranslational modifications, respectively. By using NMR, we have determined the structure of a 110-residue fragment of murine Ets-1 that includes the PNT domain and MAP kinase site. The Ets-1 PNT domain forms a monomeric five-helix bundle. The architecture is distinct from that of any known DNA- or protein-binding module, including the helix-loop-helix fold proposed for the PNT domain of the ets protein TEL. The MAP kinase site is in a highly flexible region of both the unphosphorylated and phosphorylated forms of the Ets-1 fragment. Phosphorylation alters neither the structure nor monomeric state of the PNT domain. These results suggest that the Ets-1 PNT domain functions in heterotypic protein interactions and support the possibility that target recognition is coupled to structuring of the MAP kinase site.

Structural module that acts in a biological context. To create a framework for understanding the role of the PNT domain in kinases or the acute myeloid leukemia (AML)-1B transactivation, yet protein TEL with segments of genes encoding several tyrosine domain is implicated in the self-association of chimeric oncoprotein Ets-1, Ets-2, GABP*, and TEL from vertebrates, and PNT-PZ and Yan from Drosophila (Fig. 1). The PNT domain occurs in approximately one-third of the ets proteins, including Ets-1, Ets-2, GABPs, and TEL from vertebrates, and PNT-P2 and Yan from Drosophila (Fig. 1). This domain is proposed to mediate protein-protein interactions and to be regulated by ras-dependent signaling because of the presence of an adjacent mitogen-activated protein (MAP) kinase phosphorylation site (1). In particular, the PNT domain is implicated in the self-association of chimeric oncoproteins, identified in human leukemias, that result from chromosomal translocations of the gene encoding the ets protein TEL with segments of genes encoding several tyrosine kinases or the acute myeloid leukemia (AML)-1B transcription factor (3-9).

To date sequence conservation has defined the PNT domain, yet it has not been established that this region is a structural module that acts in a biological context. To create a framework for understanding the role of the PNT domain in the regulation of a variety of ets proteins and in the oncogenic potential of TEL fusion proteins, we have characterized structurally a fragment of Ets-1 that includes this domain and the adjacent MAP kinase phosphorylation site.

MATERIALS AND METHODS

Protein Samples. DNA sequences encoding Ets-1(1-138), Ets-1(29-138), and Ets-1(51-138) were PCR-amplified from the full-length murine ets-1 cDNA and cloned into the pET28 (Ets-1(1-138)) or pET22b expression vectors (Invitrogen). The single substitutions of Leu-36 to Pro (L36P) or Thr-38 to Ala (T38A) were introduced into Ets-1(29-138) by PCR-based site-directed mutagenesis. Unlabeled and labeled Ets-1(1-138) and Ets-1(51-138) were prepared from Escherichia coli BL21 (ADE3) grown in 1-broth or minimal media, respectively, and purified by anion exchange (Mono Q; Pharmacia). Both proteins eluted with 1200 mM NaCl in 50 mM Tris, pH 8.5. Ets-1(1-138) also was purified by reversed-phase chromatography using a Vydate C-18 column. Ets-1(1-138) was isolated by metal-chelation chromatography, followed by cleavage with thrombin to remove the N-terminal His tag. Ets-1 fragments were concentrated by lyophylization or with a micro-concentration device. Samples of 1-2 mM Ets-1(1-138) were prepared for NMR analysis by dialyzing the resuspended lyophilized protein against 2 mM NH4HCO3 and 5 mM DTT (pH 8.0), followed by 10 mM KCl, 10 mM KH2PO4, and 10 mM DTT (pH 6.5). D2O (16%) was added for a deuterium lock.

A 0.8 mM sample of uniformly 15N-labeled L36P Ets-1(1-138) was phosphorylated by incubating 5 mg of protein with 2.5 mg of sea star p44 MAP kinase (Upstate Biotechnology, Lake Placid, NY) and 10 mM ATP in buffer (12 mM Tris/1 mM Glycerol phosphate/8 mM MgCl2/0.5 mM sodium vanadate/2 mM DTT, pH 7.2) at 16°C for ~2 days (10). The reaction was monitored by electrospray ionization mass spectrometry and, upon completion, the [31P,15N]-labeled fragment was repurified by reversed-phase HPLC.

Protein Characterization. Sedimentation equilibrium analysis of Ets-1(1-138) was performed by using a Beckman Model E ultracentrifuge operating at 22,000 rpm. The sample was 0.17 mM in 100 mM KCl, 25 mM KH2PO4, 10 mM DTT at pH 6.5 and 20°C. Size exclusion chromatography was carried out by using a HiLoad 16/60 Superdex 75 prep grade column (Pharmacia) with samples in 20 mM citrate, 130 mM KCl, and 10 mM DTT at pH 6.0 and 4°C. Protein stability was measured by using a Jasco (Easton, MD) J-720 spectropolarimeter (11).
Monitor the CD signal at 222 nm as a function of temperature. Samples were 20 μM protein (5 mM sodium phosphate, 5 mM sodium citrate, 5 mM sodium borate, 5 mM DTT), without or with 4 M urea at pH 6.5 in a 0.1-cm water-jacketed quartz cuvette. The urea was added to shift the unfolding transition to lower temperatures. Partial proteolysis of Ets-1 (29-138) (2.75 μg) in 20 mM citrate, 150 mM KCl, and 1 mM DTT was carried out by incubation with 200 ng of trypsin for 2 min at pH 6.0 and 25°C, as described previously (11).

NMR Spectroscopy. NMR experiments were carried out at 30°C on a Varian Unity 500 NMR spectrometer equipped with a pulsed-field gradient accessory. Data were processed by using NMRpipe (2) and NMRpredictor (13). Essentially complete H, 13C, and 15N spectral assignments of Ets-1 (29-138) were obtained from an extensive set of heteronuclear NMR spectra (14). The diastereotropic methyls groups of valine and leucine were stereospecifically assigned by using biosynthetically directed 13C labeling (15).

Resonances from the aromatic residues in Ets-1 (29-138) were identified, and both histidines were shown to be in the neutral tautomeric form at pH 6.5, as described elsewhere (16). Assignment of the amide resonances in the unmodified and phosphorylated forms of L36P Ets-1 (29-138) were obtained by using 15N-total correlation spectroscopy (TOCSY)/nuclear Overhauser effect spectroscopy (NOESY)-heteronuclear single quantum correlation (HSQC) experiments. 15N Ti, T2, and heteronuclear NOE relaxation data were recorded and analyzed as described by Farrow et al. (17).

Figure 1. The PNT domain is defined by a region of sequence conservation found in a subset of eae proteins. (A) Schematic diagram of the murine Ets-1 protein showing the locations of the PNT domain, MAP kinase phosphorylation site (Thr-38*), and DNA-binding ETS domain with flanking autoinhibitory sequences (1). The central region of the protein contains putative transcriptional domains. (B) Alignment of the sequences of PNT domains and preceding N-terminal regions from the eae family members murine Ets-1, Ets-2, GABPs, and Fli-1, human Egr, Tel, and Ese, and Drosophila PNT-P2, E1g, and Yan. The positions of highly conserved amino acids are highlighted in black (seven or more members having Blosum62 substitution scores ≥1), and of those moderately conserved residues in gray (six or more members having Blosum62 substitution scores ≥0). The MAP kinase phosphorylation sites identified in Ets-1, Ets-2, and PNT-P2 are boxed. Based on a consensus MAP kinase substrate sequence P-X-T/E, Tel also contains a potential phosphorylation site (underlined). Positions of tryptic cleavage in Ets-1 (29-138) under conditions of partial proteolysis are denoted by v. The five α-helices (cylinders) in the Ets-1 PNT domain were identified by NMR methods. The fractional solvent accessibility of the side chains in a low-energy structure of Ets-1 (29-138) are illustrated (cone = 0–25%).

Structure Determination. An ensemble of 28 Ets-1 (29-138) structures was computed from 1,568 distance (877 intrareidue, 309 sequential, 175 medium range, and 207 long range), 34 hydrogen-bonding, and 167 dihedral angle (65 φ, 70 χ, and 32 χ) restraints by using a simulated annealing protocol with X-PLOR version 3.8 (18). Interproton distance restraints were derived from three-dimensional (3D) 15N-NOESY-HSQC, 3D simultaneous 13C/15N-NOESY-HSQC, 4-dimensional 13C,15C heteronuclear multiple quantum correlation (HMOC)-NOESY-HMQC spectra, and two-dimensional homonuclear NOESY experiments in D2O (for aromatic side chains), all recorded with T2 = 75 msec. Distances were calibrated as described previously (19). Hydrogen bonds were included as distance restraints for those amides remaining protonated 45 min after transfer of the protein to D2O buffer. Phi dihedral angles were restrained based on JHNH, HN exchange constants measured with the NHNA and HMOC-J experiments (20). Phi angles were restrained as described by Gagné et al. (21). Chi angles were restrained according to a staggered rotamer model using coupling patterns observed for stereospecifically assigned Hβφ in 15N-TOCSY-HSQC and NHNA spectra and for the methyls of Thr II, Ile, and Val in long-range 13C,15N and 13C,13C correlation spectra (20).

The X-PLOR energies for the ensemble of Ets-1 (29-138) structures are: Epress = 166.0 ± 5.3, Elocal = 7.0 ± 1.7, Eel-3 = −431.0 ± 22.8, Eens = 2.5 ± 0.6, and Emax = 19.1 ± 2.6 kcal·mol−1. No distance or dihedral angle violation was greater than 0.25 Å or 5°, respectively, and the rms deviations from the
idealized values are: bonds = 0.0040 ± 0.0001 Å, angles = 0.446° ± 0.006°, and improper angles = 0.283° ± 0.010°. Within this ensemble, 98.8% of residues have (φ, ψ) angles in the core or allowed regions of a Ramachandran plot, as determined by using PROCHECK-NMR (22). All non-glycine residues in disallowed (φ, ψ) regions are located within the disordered termini of Ets-1(29-138).

**RESULTS**

Biophysical Characterization of Ets-1(29-138). Ets-1(29-138) is a 110-residue polypeptide, encoded by exons III and IV of the ets-1 gene (23), which includes both the PNT domain and MAP kinase substrate site. The C-terminal three-quarters of this fragment, which corresponds to the FNT domain (Ser-54 to Glu-135), shares 30-65% sequence identity with nine other ets family members (Fig. 1). Ets-1(29-138), which was expressed as a soluble protein in E. coli, folds into a stable conformation with significant helical content as evidenced by a CD spectrum with pronounced minima at 208 and 222 nm (θ222 = −7,600 deg cm2 dmol-1), a well-dispersed NMR spectrum (Fig. 24), and a reversible two-state unfolding transition with a midpoint temperature of ~78°C at pH 7.0. Furthermore, Ets-1(29-138) is monomeric under a variety of experimental conditions as demonstrated by sedimentation equilibrium (MWapp = 11,400 Da; predicted = 12,553.5 Da), 15N relaxation measurements, which revealed an overall rotational correlation time of 6.9 ns (Fig. 2C), and size exclusion chromatography, in which the protein eluted as a single peak near a 17-kDa marker. Taken together, these results establish that residues 29–138 from Ets-1 include an independent structural module.

Structure Determination of Ets-1(29-138). The structure of Ets-1(29-138) was determined by NMR spectroscopy (Fig. 3). The Ets-1 PNT domain shows a novel architecture consisting of five α-helices. The C-terminal portion of this domain, which includes helices H2–H5, folds into a well-defined bundle with rms distributions about the mean coordinate positions of 0.39 ± 0.06 Å for the backbone atoms and 0.85 ± 0.06 Å for all heavy atoms of residues 63–133. Helices H3 and H4 are arranged head to tail, yet are clearly separated by a bend centered at Gly-109 such that H3 is approximately perpendicular to H2, H4, and H5. Helix H2 is antiparallel to H4, and H2 lies across H3, H4, and H5. Consistent with this tertiary fold, helices H2, H4, and H5 are amphipathic, whereas the short H3 helix participates in a minor β-sheet.

**Fig. 2.** (A) The PNT domain (Ser-54 to Glu-135) is an independently folded structural module as evidenced by well-dispersed peaks in the 1H-15N HSQC spectrum of Ets-1(29-138). Residues N-terminal to this domain, including the MAP kinase substrate site, adopt a disordered conformation with 1H chemical shifts that cluster near 8.2 ppm. Aliased peaks are identified by •. (B) Hydrogen-deuterium exchange studies identify amide protons that are protected from the solvent caused by hydrogen bonding and/or burial within Ets-1(29-138). • indicate residues with resolved 1H-15N HSQC cross peaks that have exchange rates >103 slower than expected for a random coil polypeptide. (C) NMR relaxation measurements provide information about the global tumbling and fast internal motions of Ets-1(29-138). Analysis of the amide 15N T1 and T2 lifetimes (msec) indicate residues that are protected from the solvent caused by hydrogen bonding and/or burial within Ets-1(29-138). Residues N-terminal to this domain, including the MAP kinase substrate site, adopt a disordered conformation with 1H chemical shifts that cluster near 8.2 ppm. Aliased peaks are identified by •. (D) Chemical shift perturbations indicate that the effects of the Leu-36 to Pro mutation and the subsequent phosphorylation of Thr-38 are localized to the MAP kinase substrate site in the disordered N-terminal region of Ets-1(29-138). Shown are the absolute values of the changes in the amide 1H and 15N chemical shifts (ppm) caused by the mutation and phosphorylation plotted versus residue number. The small changes observed for residues in the PNT domain reflect subtle differences in experimental conditions.
FIG. 3. The tertiary structure of Ets-I was determined by NMR methods. (A) Superimposition of the main chain atoms from 28 NMR-derived structures of Ets-I(29-138) aligned by using residues 63-133. The five α-helices in the PNT domain are colored (HI: residues 54-62; H2: 75-87; H3: 102-107; H4: 110-116; H5: 123-132), whereas the remainder of the main chain is shown in gray. The N and C termini of the molecule (residues 29-49 and 135-138) are disordered as evidenced by both high structural rms deviations and 15N NMR relaxation data. (B) Ribbon diagram of a representative low-energy structure calculated for Ets-I(29-138). Only a single conformation is shown for the flexible N and C termini. (C) A low-energy structure of residues 26-132 of Ets-I(29-138) showing the positions of the side chains that are highly conserved (Fig. 18) among the PNT domains of 10 εlS proteins (green = hydrophobic, red = acidic, blue = basic, dark gray = polar). Nonpolar (Fig. 1B). Packing of these helices is mediated by several highly conserved hydrophobic and aromatic residues including Val-77, Trp-80, Val-81, Trp-83, and Ala-84 (H2), Leu-105 (H3), Phe-113 (H4), and Ile-124, Leu-125, His-128, Leu-129, and Leu-132 (H5) (Fig. 3C). Conserved residues in the loop between H2 and H3 (Leu-90, Val-93, and Phe-98) also contribute to the hydrophobic core of Ets-I(29-138). Ets-I(29-138) contains a fifth helix (H1) that, although well defined by chemical shift, NOE, and J-coupling data, does not appear intimately associated with the core helical bundle (Fig. 3). The solvent exposure of helix H1 is consistent with its predominantly polar nature, its susceptibility to proteolysis at its C terminus (Arg-62) by trypsin, and its lack of protection against amide hydrogen exchange under the conditions examined (Figs. 1B and 2B). The position of this helix with respect to the remainder of the molecule is not precisely established, being determined by a small number of medium- and long-range NOE-derived distance restraints involving Leu-63, Ile-65, Pro-66, Pro-69, and Trp-72 in the loop between H1 and H2. Nevertheless, the helix itself is well defined locally, with an rms deviation of 0.53 ± 0.12 Å for the main chain atoms and 1.4 ± 0.15 Å for the heavy atoms of residues 54-62. 15N NMR relaxation measurements also indicate that the backbone of H1 is well ordered (Fig. 2C). Furthermore, CD and NMR spectroscopic measurements demonstrate that this helix unfolds cooperatively with the remainder of Ets-I(29-138) (not shown). These results indicate that helix H1, although exposed to the solvent, is an integral structural component of the Ets-I PNT domain.

Inspection of the Ets-I PNT domain structure reveals several potential protein binding sites. Protein–protein associations of both hydrophobic and electrostatic/hydrogen bonding interactions between interfaces composed of complementary nonpolar and charged/polar residues. A common surface of interactive sites contains a hydrophobic patch surrounded by polar groups (24). One such surface in Ets-I(29-138), formed by helices H4 and H5, displays a hydrophobic region, centered around Trp-126, that is encircled by six acidic side chains (Fig. 4A). Similarly, the surface formed by helix H3 and the preceding loop from H2 contains several exposed hydrophobic residues that are surrounded by charged glutamates and lysines (Fig. 4B).

The region of Ets-I(29-138) that precedes helix H1 is disordered. These amino acids display random coil amide H4 and 15N chemical shifts (Fig. 2A) and very high rms deviations within the ensemble of calculated structures (Fig. 3B). Conformational mobility also is detected through 15N relaxation.
measurements (Fig. 2C). Consistent with these NMR results, the region is readily susceptible to tryptic cleavage, specifically at Lys-42 and Lys-56 (Fig. 1B). Furthermore, deletion of these residues to produce Ets-1(1-138) does not perturb the $^{1}H$ and $^{15}N$ chemical shifts of the remaining amides that form helices H1-H5. Similar NMR measurements reveal that the first 28 residues of Ets-1(1-138) also are disordered (not shown). Together, these results demonstrate that the N-terminal ~50 residues of Ets-1 are highly flexible in solution and structurally independent of the PNT domain, at least in the context of Ets-1(1-138) and Ets-1(29-138). The observation that this sequence is sensitive to tryptic cleavage in experiments performed on full-length Ets-1 (11) suggests that these residues also are disordered in the native protein.

Phosphorylation of Ets-1(29-138). The function of Ets-1 in transcription assays is enhanced by ras-dependent signaling that requires a single MAP kinase substrate site, Leu-Leu-Thr-Pro (25-27). This site is located within the flexible, nonstructured region of Ets-1 (Fig. 3A and B). To provide a foundation for investigating the possible mechanisms by which phosphorylation regulates the function of Ets proteins, we characterized the effects of this posttranslational modification on the structural and dynamic properties of Ets-1(1-138). Ets-1(29-138) was phosphorylated by using active sea star p44 MAP kinase. To increase the efficiency of this enzymatic reaction, Leu-36 was replaced with a proline in Ets-1(29-138). This substitution generated an optimized MAP kinase substrate sequence (Pro-Leu-Thr-Pro) (10, 28), which matched the site in Drosophila PNT-P2 (29), and led to the enhanced phosphorylation of Thr-38. The posttranslational modification was verified by mass spectrometry, which showed the expected increase of 80 Da, and by $^{31}P$NMR measurements. Mutation of Thr-38 to Ala completely prevented phosphorylation of Ets-1(29-138), confirming that Thr-38 is the only MAP kinase phosphorylation site. This substitution generated an optimized MAP kinase substrate sequence (Pro-Leu-Thr-Pro) (10, 28), which matched the site in Drosophila PNT-P2 (29), and led to the enhanced phosphorylation of Thr-38. The posttranslational modification was verified by mass spectrometry, which showed the expected increase of 80 Da, and by $^{31}P$NMR measurements. Mutation of Thr-38 to Ala completely prevented phosphorylation of Ets-1(29-138), confirming that Thr-38 is the only MAP kinase phosphorylation site.

Our biological studies demonstrate that Ets-1(29-138), both phosphorylated and unphosphorylated, is a monomer. Like­wise, sedimentation equilibrium studies demonstrate that unphosphorylated full-length Ets-1 is monomeric in solution (J.S. and L. Joss, unpublished work). These results argue that the PNT domain from Ets-1 may bind to a heterotypic PNT domain(s) or may function through intra- or intermolecular interactions unrelated to protein-association modules. The structure of Ets-1(29-138) provides important clues for ongoing studies aimed at defining the potential targets for the PNT domains of Ets family members.

Distinct patterns of protein-protein interactions involving specific eTS transcription factors could arise from sequence variability among the PNT domains. As would be expected for a structural fold, the most highly conserved positions lie within the hydrophobic core (Figs. 1B and 3C). On the other hand, the most variable positions map to the surfaces of the domains, particularly on helices H1, H3, and H4 and in the extended loop linking H2 and H3, and to the disordered region including the MAP kinase phosphorylation site. The hydrophobic and charged groups forming the postulated association surfaces of Ets-1 (Fig. 4) are not strictly conserved among PNT domains, thus providing the potential for specific interactions with target proteins. Other possible sources of variation include a four-residue insertion between the predicted positions of helices H1 and H2 in the PNT domains from TEL and Yan (Fig. 1F). These insertions may alter or disrupt the packing of the exposed helix H1 against the core helical bundle or provide additional interprotein contacts. Finally, because of the low sequence conservation within the region corresponding to H1 in the Ets-1 fragment, it is plausible that this helix is not present in all eTS PNT domains. The flexibility or absence of helix H1 could produce an additional hydrophobic interaction surface by exposing several conserved aromatic side chains corresponding to Trp-72, Trp-90, and Trp-85 in Ets-1 (Fig. 1C). Phosphorylation of sequences adjacent to the PNT domain provides another potential source for specificity and regulation. Although phosphorylation enhances the transactivation function of Ets-1, Ets-2 and PNT-P2, the precise mechanism by which this occurs is unknown (25-27, 29). Our analysis of Ets-1(29-138) indicates that the MAP kinase substrate site lies within a flexible segment of this Ets-1 fragment (as well as that of Ets-1(1-138)), and that phosphorylation of Thr-38 does not
change its structural or dynamic properties. These results imply that binding of this site by potential partner transcription factors, perhaps in conjunction with the PNT domain, is coupled to the ordering of these residues. This type of a regulated folding event is exemplified by the phosphorylation-dependent association of the KIX domain of the cAMP-regulated transcription factor CREB with its coactivator CREB binding protein (CBP). The KIX domain undergoes a random coil-to-helix transition that is induced by CBP binding, and not by phosphorylation alone (36).

In conclusion, the description of the Ets-1 PNT domain and its adjacent MAP kinase substrate site establishes this region of conserved sequence as a structural module, that in the case of Ets-1, is not affected by phosphorylation. The PNT domain, which is clearly unrelated to the helix-loop-helix motif, constitutes one of the few non-DNA binding domains of transcription factors whose structure has been characterized. The conserved and variable features of the PNT domain could accommodate either self-association or heteroteric interactions of specific egr family members.

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EXPRESSION, PURIFICATION, AND CHARACTERIZATION
OF PNT DOMAIN CONTAINING FRAGMENTS OF TEL
Introduction

The ets family member TEL plays important roles in both normal biological functions and human malignancies. Mice homozygous for null alleles of TEL die at embryonic day 10.5-11.5 and have defects in yolk sac angiogenesis as well as specific areas of apoptotic cell death (Wang et al., 1997). In adults, TEL is required for bone marrow hematopoiesis, as demonstrated by studies of mouse chimeras made with TEL<sup>-/-</sup> embryonic stem cells (Wang et al., 1998). Naturally occurring disruptions of the TEL gene have deleterious affects on cells in the context of human leukemias. For instance, the TEL gene is involved in chromosomal translocations associated with leukemias that result in expression of fusion proteins containing part of the TEL protein (Rowley, 1998). The TEL PNT domain is fused to the receptor tyrosine kinase platelet derived growth factor receptor β (PDGFRβ) (Golub et al., 1994) in chronic myelomonocytic leukemia, JAK2 in acute lymphoblastic leukemia (ALL) (Lacronique et al., 1997), and ABL in acute myeloid leukemia (Papadopoulos et al., 1995; Golub et al., 1996), and the transcriptional activator AML1/CBFA2 in 20% of cases of ALL (Golub et al., 1995) (Fig. 3.1). Understanding the function of the TEL protein and PNT domain will provide insight into biological and tumorigenic roles of this ets family member.

Self-association of the PNT domain appears to be required for the oncogenic activity of the fusion proteins. Normally, the activity of receptor tyrosine kinases is regulated by ligand binding that results in oligomerization and activation of the tyrosine kinase catalytic domain. In the fusion protein, however, the TEL PNT domain replaces the oligomerization motifs. Constitutive oligomerization of the PNT domain of TEL results in constitutive activation of the catalytic domains of PDGFRβ, ABL, and JAK2.
**Figure 3.1** TEL fusion proteins. The sequence of TEL involved in the fusion protein is shown. Functional domains of the fusion partner are described. The associated leukemia is listed to the right.
(Carroll et al., 1996; Golub et al., 1996; Jousset et al., 1997; Lacronique et al., 1997). The constant signaling mediated by oligomerization of the PNT domain is thought to be a critical event in oncogenesis. Oligomerization of the PNT domain is also observed in the TEL-AML1/CBFα fusion protein, and the PNT domain plays an important role in the converting AML1 from an activator of transcription to a repressor (Hiebert et al., 1996; Lopez et al., 1999).

The PNT domain of TEL is also involved in the function of TEL as a transcriptional repressor (Chakrabarti and Nucifora, 1999; Lopez et al., 1999). There are two models for the role of the PNT domain in repression. One model postulates that PNT domain mediated oligomerization facilitates binding of transcriptional corepressors outside the PNT domain (Lopez et al., 1999; Uchida et al., 1999). A second model proposes that the PNT domain is a modular repression domain that interacts directly with transcriptional corepressors. Evidence exists that the TEL PNT domain may interact with the corepressor mSin3A (Fenrick et al., 1999; Wang and Hiebert, 2001), but this is a matter of controversy.

Beyond oligomerization, other protein partners and functions for the PNT domain of TEL are not well-established. First, two-hybrid analysis indicated that a PNT domain containing fragment of TEL PNT oligomerizes with a PNT domain containing fragment of Fli-1 (Kwiatkowski et al., 1998). It was not determined, however, whether the PNT domain of either protein was necessary or sufficient for the interaction. Second, the PNT domain of TEL is required for an interaction with the highly related protein TEL2 (Potter et al., 2000), but the significance of this interaction is not known. Third, the PNT domain of wild-type TEL and TEL-AML is reported to
interact with the SUMO conjugating enzyme UBC9 (Chakrabarti et al., 1999; Chakrabarti et al., 2000). This binding event results in SUMO modification of the PNT domain and alteration of nuclear localization of TEL (Chakrabarti et al., 2000). Of the potential partners, the data is most convincing for the UBC-9 interaction. It is not known how oligomerization affects these protein interactions.

Additional support for the oligomerization of the TEL PNT domain is provided by structural studies of the sterile-α-motif (SAM) domain. SAM domains are found in diverse sets of proteins, including cell surface receptors, yeast mating pathway proteins, and polycomb genes (Schultz et al., 1997). Several SAM domains are also reported to mediate homotypic and heterotypic associations. The EphA4-SAM domain was a dimer in crystallographic studies (Stapleton et al., 1999), and the EphB2-SAM domain showed a polymeric structure (Smalla et al., 1999; Thanos et al., 1999b). However, the EphB2-SAM domain was also determined to be a monomer in a crystallography experiment, and biophysical techniques suggest it is monomeric at high concentrations (Thanos et al., 1999a). The SAM domains of the polycomb transcriptional repressors polyhomeotic (ph) and Sex comb on midleg (Scm) can self-associate as well as interact with each other (Peterson et al., 1997). These studies lend support to a role for the TEL PNT domain in self-association.

Although various assays demonstrate homotypic interactions for the TEL PNT domain, the exact oligomerization state of the TEL PNT domain was unclear at the commencement of this thesis work due to the nonbiophysical nature of the reported experiments. Knowledge of the oligomerization state has implications for the mechanism of TEL PNT domain mediated repression and its role in the genesis of
leukemias. To determine the oligomerization state of the TEL PNT domain, an *in vitro* biochemical approach was undertaken. Murine TEL and several PNT domain containing fragments of TEL were overexpressed in bacteria. Characterization of the TEL fragments demonstrated that all proteins were predominantly insoluble. Several TEL fragments exhibited slight solubility under native conditions, and size exclusion chromatography indicated that these proteins were multimeric, although an exact oligomerization state could not be determined. These findings are consistent with a report published subsequent to the completion of this work demonstrating that the TEL PNT domain forms a helical polymer (Kim et al., 2001).

**Materials and methods**

Construction of bacterial expression plasmids

Plasmids encoding murine TEL(1-126), TEL(12-126), and TEL(60-126) were generated by PCR amplification of cDNA encoding murine TEL (pBKS-murine TEL; generous gift of O. Bernard (Poirel et al., 1997)) followed by subcloning into the *Nde I* and *Hind III* sites of pET-22b(+). A plasmid encoding full-length murine TEL (pET-22b(+)-murine TEL) was generated as follows: pET-22b(+)-murine TEL(1-126) was digested with *BstB I*, which cleaves a site in the TEL ORF, and *Hind III*, which cleaves a site in the polylinker. The cDNA encoding murine TEL was digested with *BstB I* and *Hind III*, which released fragments of 691, 1998, and 2947 bp. The 1998 bp fragment was ligated into the *BstB I* and *Hind III* sites of pET-22b(+)-murine TEL(1-126). A second bacterial plasmid encoding full-length TEL was generated by restriction digest of pET-22b(+)-murine TEL with *Nde I* and *Hind III* followed by subcloning of the TEL ORF into the *Nde I* and *Hind III* sites of the pAED4 plasmid. A plasmid encoding full-
length histidine-tagged TEL (pET5H-murine TEL) was generated by restriction digest of pET-22b(+)murine TEL with \textit{Nde} I and \textit{Hind} III followed by subcloning into the \textit{Nde} I and \textit{Hind} III sites of pET-22b(+) engineered to encode an N-terminal five histidine tag (generous gift of L. McIntosh). A plasmid encoding histidine/heart muscle kinase (HMK)/FLAG-tagged murine TEL was constructed by ligating duplexed oligonucleotides encoding HMK and FLAG tags into the \textit{Nde} I site of pET5H-murine TEL.

**Protein expression and purification**

Plasmids encoding TEL proteins were transformed into BL21(DE3) cells with or without the pLysS plasmid. Cells were grown in Luria broth (LB) containing 125 µg/ml ampicillin with or without 20 µg/ml chloramphenicol at 22.5°C, 30°C, or 37°C. At an $A_{600}$ of 0.6-0.9, protein expression was induced by the addition of isopropyl-$\beta$-D-thiogalactosidase (IPTG) to a final concentration of 1 mM, and growth was allowed to proceed for 3 hr. To test solubility, cells were resuspended in 50 mM Tris-HCl, pH 7.9, 2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1 mg/ml lysozyme, and then sonicated. Insoluble material was pelleted by centrifugation. Protein solubility was also tested in 25 mM Tris-HCl, pH 7.9, 10% glycerol, 0.1 mM EDTA, and 100 mM KCl.

**Size exclusion chromatography**

Fragments of TEL were chromatographed on a Superose 12 size exclusion column (Amersham-Pharmacia) equilibrated with 20 mM Tris-HCl pH 7.9, 10% (vol/vol) glycerol, 10 mM MgCl$_2$, 1 mM EDTA, and 100 mM KCl and calibrated with BioRad gel filtration standard. Fractions of 1 ml were collected, TCA precipitated, and
proteins were electrophoresed and detected by silver staining. Full-length TEL proteins were chromatographed on a Superdex-75 or Sephacryl-200 size exclusion column (Amersham-Phannacia).

**Limited proteolysis**

TEL proteins were incubated with various amounts of trypsin or chymotrypsin at room temperature for 2 min. Reactions were stopped by the addition of 3x SDS sample buffer and heating at >95°C for 5 min. Reactions products were electrophoresed on 15% polyacrylamide gels and detected by staining with Coomassie-Blue.

**Results**

**Fragment design**

To generate proteins for *in vitro* studies, constructs were designed to express PNT domain containing fragments of TEL in bacteria. The N- and C-terminal boundaries for three fragments were selected based on the position of secondary structural elements in the PNT domain of Ets-1 (Fig. 3.2; Slupsky et al., 1998). TEL(1-126) spans the N-terminus through the PNT domain (residues ~40-121), TEL(12-126) mimics the Ets-1(29-138) fragment and has a shortened N-terminal extension, and TEL(60-126) contains sequence that is conserved with helices H2-H5 of the Ets-1 PNT domain. The sequence comprising helix H1 of the Ets-1 PNT domain is not well-conserved in TEL and may not form a helix in TEL. The TEL(60-126) fragment also resembles a minimal fragment of TEL reported to oligomerize with itself (residues 59-119 of TEL) (Jousset et al., 1997).
Figure 3.2 TEL fragments. Ets-1(29-138) is shown for comparison with the position of alpha helices (white boxes) noted. The asterisk denotes the MAPK phosphorylation site in Ets-1, which has not been demonstrated to be functionally conserved in TEL.
Protein expression and characterization

To generate proteins for biochemical characterization, the TEL PNT domain containing fragments were overexpressed in *E. coli* BL21(DE3) cells at 37°C. Overexpression of each fragment was detected by staining SDS-polyacrylamide gels with Coomassie Blue (Fig. 3.3). Protocols were followed to obtain soluble protein; however, a majority of each fragment was insoluble under native conditions (data not shown).

Soluble protein is necessary for biochemical and biophysical characterization of the oligomerization state of a protein. It was speculated that the aggregates of TEL formed due to hydrophobic interactions among unfolded proteins. Under this hypothesis, proteins were assumed to have been expressed in bacteria too rapidly to achieve their thermodynamically favored conformation. Mechanistically, hydrophobic patches of one monomer are entropically driven to interact with hydrophobic patches on another monomer, ultimately resulting in inclusion body formation. Given the assumption, these forces must be overcome to obtain soluble protein.

One method used to favor expression of a soluble protein within bacteria is to modulate the temperature at which protein expression occurs. A lower temperature of expression can lead to enhanced protein solubility by decreasing the rate of protein production, thereby allowing a protein time to achieve its native fold. Expression of TEL PNT domain containing fragments at 30°C, however, did not affect their solubility (data not shown).

Another strategy to obtain soluble protein is to denature the insoluble protein and then remove the denaturant by dialysis. To test the feasibility of this strategy,
Figure 3.3 Overexpression of TEL fragments. Shown is extract from uninduced (U) and induced (I) cells electrophoresed on SDS-polyacrylamide gels that were stained with Coomassie Blue. Arrowheads demarcate overexpressed TEL proteins. Molecular masses are noted in kDa. Each fragment is identified above the corresponding gel.
insoluble TEL fragments were dissolved in a buffer containing urea (5M) and then dialyzed directly into buffer lacking urea. The strategy did not succeed, as the TEL fragments precipitated during dialysis. It was hypothesized that the proteins precipitated because the urea was removed too rapidly to allow proper refolding. This possibility was addressed by removing urea in a step-wise manner. Unfortunately, successive dialysis of TEL fragments into buffers containing 3 M and then 1 M urea, followed by removal of urea, also led to precipitation of the TEL fragment.

The concentration of a protein during refolding can affect its ability to assume its native fold. Lower concentrations typically increase the likelihood of obtaining soluble protein. However, diluting TEL fragments at the start of dialysis to concentrations of 1.8 mg/ml, 0.9 mg/ml, and 0.18 mg/ml, still resulted in quantitative precipitation following removal of denaturant.

It was found by chance that a fraction of insoluble TEL(12-126) could be solubilized by washing the TEL inclusion body with buffer lacking denaturants. To determine its oligomerization state, this solubilized protein was subjected to size exclusion chromatography on a Superose 12 column. The TEL fragment eluted from the column over a wide range of estimated molecular masses, suggesting that TEL existed in several multimeric states (Fig. 3.4).

Biochemical characterization of full-length TEL

It was possible that inclusion body formation and precipitation occurred because sequences outside the PNT domain of TEL are required for proper folding of the domain. This possibility was addressed by using partial proteolysis to determine the domain structure of full-length TEL. Under limiting conditions, proteases preferentially
Methodology

- Solublize TEL(1-126)
- Superose 12
- Collect fractions
- TCA ppt
- SDS-PAGE
- Silver stain

**Figure 3.4** Size exclusion chromatography of TEL(1-126). (Left) Methodology. (Right) SDS-polyacrylamide gel of fractions from the Superose 12 size exclusion column; gel is silver stained. Estimated range of molecular masses of proteins eluted from the size exclusion column is noted above individual lanes. TEL(12-126) is indicated by the arrowhead.
cleave proteins in sequences that lie outside well-folded regions and secondary structural elements. These sites are expected to define domain boundaries, and the resulting fragments are predicted to be soluble when overexpressed.

To generate substrates for partial proteolysis, four bacterial expression plasmids encoding full-length TEL were constructed. These included two constructs for untagged, full-length murine TEL (pET22b(+) vector backbone and pAED4 vector backbone), TEL containing an N-terminal five histidine tag (5H-TEL), and TEL with three consecutive N-terminal tags (5H/HMK/FLAG) (Fig. 3.2). The 5H tag facilitates biochemical purification, the HMK (heart muscle kinase) tag can be used to radiolabel the protein by phosphorylation with protein kinase A, and the FLAG tag both facilitates purification and serves as an epitope that can be recognized by an anti-FLAG antibody.

Full-length TEL proteins were overexpressed in BL21(DE3) cells. Each protein was expressed, albeit at lower levels compared to the PNT domain containing fragments (Fig. 3.3). Unfortunately, like the TEL PNT domain containing fragments, TEL and 5H-TEL were predominantly insoluble when expressed at 37°C (data not shown). Expression of untagged TEL at 22.5°C and 30°C did not detectably alter its solubility, and solubilization of TEL in a buffer containing urea (6M) followed by dialysis into buffers containing different concentrations of KCl (50 mM, 100 mM, or 500 mM) did not yield detectable increases in soluble protein (data not shown).

Studies were next focused on characterization of 5H-TEL, which could be purified and concentrated more easily due to the histidine tag. Insoluble 5H-TEL purified on a nickel-chelating column under denaturing conditions (8M urea) was dialyzed into buffer lacking urea. Following dialysis, approximately 50% of the TEL
protein was soluble. To estimate its oligomerization state, 5H-TEL was passed over a Sephacryl-200 size exclusion column. Most 5H-TEL protein eluted in the void volume, indicating that the protein existed as a multimer (data not shown). To determine if detergent would assist in refolding out of urea, insoluble 5H-TEL was solublized in urea (6M) and sodium dodecyl sulfate (SDS) (0.2%), dialyzed into a buffer lacking urea and SDS, and passed directly over a Sephacryl-200 column. 5H-TEL prepared in this manner also eluted in the void volume (data not shown). To determine if the multimeric 5H-TEL was folded into discrete domains, the domain structure of this protein was analyzed by partial proteolysis with trypsin and chymotrypsin. A ladder of fragments of multiple sizes was generated (Fig. 3.5), suggesting that the protein may have been unfolded and did not have structured domains. However, several stable fragments did appear at higher concentrations of trypsin, suggesting that at least some of the 5H-TEL was folded or folded upon digestion.

It was possible that in vitro refolding strategies for obtaining soluble TEL failed because the urea did not completely denature the TEL proteins in the inclusion body. To determine the oligomerization state of TEL in the presence of denaturant, 5H-TEL was solubilized in a buffer containing urea (5.7 M) and subjected to size exclusion chromatography on a Sephacryl-200 column in the same buffer. As under native conditions, 5H-TEL in the presence of urea eluted from the column at the void volume (data not shown). This suggested that TEL was not unfolded by denaturants or that urea does not dissociate a tightly bound multimeric state.

Proteins can be coexpressed with chaperones to enhance their solubility. Chaperones provide an isolated environment that allows a protein to achieve its
Figure 3.5 Partial proteolysis of 5H-TEL. 5H-TEL was incubated with the indicated amounts of protease for 2 min at room temperature. Chymotrypsin (c); trypsin band (t). Molecular masses are indicated in kDa. SDS-polyacrylamide gels were stained with Coomassie Blue.
thermodynamically favored conformation. Plasmid encoding 5H-TEL was co-
transformed into BL21(DE3) cells with the plasmid pGroESL, which encodes the
bacterial chaperones GroES and GroEL. The presence of the pGroESL plasmid
appeared to slightly enhance the proportion of soluble TEL that was expressed (data not
shown). This protein was not further characterized.

Discussion

The studies described here demonstrate that full-length TEL or fragments of
TEL containing the PNT domain form inclusion bodies when expressed in bacteria.
Solubility of TEL fragments was almost completely dependent on the presence of
denaturant. Strategies designed to denature and refold the TEL PNT domain were not
successful in generating soluble protein in the absence of denaturant. Furthermore,
expressing proteins under a variety of conditions that favor solubility were also
unsuccessful.

Significant insight into the oligomerization state of the TEL PNT domain was
provided in a report by Kim et al. (2001). In this study, a point mutation was identified
by random mutagenesis that led to soluble TEL PNT domain in bacteria.
Crystallographic studies showed that this mutant version of the TEL PNT domain has a
fold similar to the Ets-1 PNT domain (Chapter 2; Slupsky et al., 1998). However,
unlike the Ets-1 PNT domain, which is monomeric, the mutated TEL PNT domain
forms a helical polymer. The wild-type TEL PNT domain also appears to form a helical
polymer, as suggested by electron microscopy.

Polymerization of the TEL PNT domain can explain several of the results
observed in this thesis work. First, the PNT domain may have formed inclusion bodies
in bacteria and precipitated following removal of denaturants not due to aggregation but by forming large polymers. Second, solubilized forms of the TEL PNT domain that eluted from the size exclusion column at a range of molecular masses (Fig. 3.4) may have been helical polymers composed of different numbers of PNT domain subunits.

Polymerization of the TEL PNT domain appears to play an important role in transcriptional repression and oncogenesis. It has not been determined whether a heterologous oligomerization motif can replace the TEL PNT domain in the context of the oncogenic translocations. As for transcriptional repression, Kim et al. (2001) proposed that polymerization of the PNT domain serves as a mechanism for spreading repressive TEL proteins along the DNA. Interestingly, spreading behavior is observed for the polycomb group of transcriptional repressors (Mahmoudi and Verrijzer, 2001). A subset of these proteins contains the related SAM domain. The SAM domains of Sex combs on midleg (Scm) and polyhomeotic (ph) form heterotypic and homotypic associations (Kyba and Brock, 1998; Peterson et al., 1997). However, these domains have not been shown to polymerize. More detailed studies are required to mechanistically understand the role of the TEL PNT domain in transcriptional repression.

Polymerization might be a feature of the PNT domains of other ets proteins. The TEL2 PNT domain, which is 62.5% identical to the TEL PNT domain (Potter et al., 2000), may form helical polymers, as the residues that form the TEL PNT domain dimerization interface are conserved in TEL2. Interestingly, no other PNT domain has been reported to polymerize, and it is clear that some PNT and SAM domains are monomeric under a variety of assay conditions (Chapter 2; Slupsky et al., 1998). It is
also noteworthy that SAM domains that are reported to form oligomers used interfaces that are distinct from those used by the TEL PNT domain for oligomerization (Stapleton et al., 1999; Thanos et al., 1999b). This suggests that individual PNT and SAM domains may have evolved the ability to oligomerize independently and not through a common ancestor.

References


CHAPTER 4

IDENTIFICATION OF PROTEIN PARTNERS OF ETS-1(29-138)  

BY AFFINITY CHROMATOGRAPHY
Introduction

Ets-1 is proposed to regulate the transactivation of multiple genes that play diverse biological roles. Through analysis of enhancer elements, Ets-1 has been implicated in mediating transactivation of genes that regulate the extracellular matrix (Trojanowska, 2000), apoptosis (Teruyama et al., 2001), and HIV proteins (Sieweke et al., 1998). Studies of mice with null alleles also demonstrate an importance for ETS-1 in hematopoiesis, as mice targeted for disruption of ETS-1 have defects in T cells and B cells (Muthusamy et al., 1995) and lack natural killer (NK) (Barton et al., 1998) and NK T cells (Walunas et al., 2000). To understand how these processes are regulated, the molecular function of the Ets-1 protein must be investigated.

The ETS-1 gene encodes a protein of 440 amino acids that contains a PNT domain and ETS domain. Many of the molecular functions of the Ets-1 protein have been studied by examining the activities of these domains independently. The DNA binding ETS domain, for instance, has a winged-helix-turn-helix structure (Donaldson et al., 1996) that preferentially binds the sequence 5'-GGA(A/T)-3' (Nye et al., 1992). Its affinity for DNA is regulated by other features, including autoinhibitory sequences (Graves et al., 1998), phosphorylation (Rabault and Ghysdael, 1994; Cowley and Graves, 2000), and protein partnerships (Goetz et al., 2000; Gu et al., 2000). These molecular features may influence the target specificity of Ets-1 in vivo.

In contrast to the ETS domain, the function of the PNT domain is less well understood. Structurally, the PNT domain is composed of five α-helices that assume a globular fold (Chapter 2; Slupsky et al., 1998). Functionally, several protein partners of the Ets-1 PNT domain have been proposed, but none are firmly established. For
instance, two-hybrid analysis suggested that a fragment of Ets-1 that contains the PNT domain interacts with the SUMO-conjugating enzyme UBC9 (Hahn et al., 1997), but this interaction was not localized to the PNT domain. Second, a fragment of Ets-1 containing the PNT domain was reported to interact with the transcriptional repressor Daxx (Li et al., 2000). Again, however, it was not determined whether the PNT domain was necessary or sufficient for the UBC9 interaction or the Daxx interaction. Therefore, the Ets-1 PNT domain has no established protein partners.

The molecular function of a MAPK site N-terminal to the PNT domain in Ets-1 is also not clear. The MAPK phosphorylation site (T38) is conserved in Ets-2 (T72) and the Drosophila ets protein Pnt-P2 (T151). The MAPK site lies on a flexible extension N-terminal to the PNT domain and can assume multiple conformations with respect to the domain (Slupsky et al., 1998). Functionally, phosphorylation of this site results in increases in the transactivation activity of Ets-1, Ets-2, and Pnt-P2 (Brunner et al., 1994; O'Neill et al., 1994; Yang et al., 1996). In Drosophila, phosphorylation of the T151 is required for normal R7 photoreceptor development (Brunner et al., 1994; O'Neill et al., 1994). However, the molecular mechanisms by which phosphorylation enhances transactivation is not understood.

Phosphorylation of Ets-1 and Ets-2 is regulated by the RAS-RAF-MEK-ERK signaling pathway (Yang et al., 1996). In vitro and in vivo studies demonstrate that the mitogen-activated protein kinases (MAPKs) ERK1 and ERK2 can phosphorylate T38 and T72 in Ets-1 and Ets-2, respectively. The highly related MAPK Rolled phosphorylates Pnt-P2 in Drosophila (Brunner et al., 1994; O'Neill et al., 1994). The MAPK JNK, which is activated by a different MAPK pathway, has also been
implicated as a physiologically relevant kinase for Ets-2 phosphorylation (Smith et al., 2000). There are no reports of other MAPKs that can phosphorylate this conserved site. The PNT domain and MAPK site may function by mediating protein interactions. Knowledge of these protein partners would help define the molecular mechanism of Ets-1 transcription factor function. To identify protein partners of the Ets-1 PNT domain and MAPK site, an affinity chromatography experiment was performed using Ets-1(29-138) as a ligand. Because Ets-1 is expressed in T cells, which reside in the thymus, a calf thymus nuclear extract was used as a source of potential partners. In the affinity chromatography experiment, over a dozen proteins from the nuclear extract interacted with the Ets-1(29-138) column. Two proteins previously proposed to interact with Ets-1 were identified: the mitogen-activated protein kinase ERK2 and the SUMO conjugating enzyme UBC9. The interaction with ERK2 was further investigated as described in Chapter 5.

Materials and methods

Expression and purification of Ets-1(29-138)

Ets-1(29-138) was overexpressed from the pET22b(+) murine Ets-1(29-138) plasmid (Slupsky et al., 1998) in BL21 (DE3) cells. A glycerol stock was used to inoculate 50 ml Luria Broth (LB) containing 125 μg/ml ampicillin and 0.08% glucose for growth overnight at 37°C. The overnight culture was subcultured i/200 in LB containing 125 μg/ml ampicillin. At an A₆₀₀ of 0.6, protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM. Cells were grown for four hr following induction. Cells were harvested, resuspended in 50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.2 mM PMSF, and 1 mM DTT,
lyzed by sonication (3 X 1 min, power level 7.5, macrotip), and soluble extract was cleared by ultracentrifugation (40,000 rpm, 45 min, 4°C, 50.2 Ti rotor) and filtration. Soluble extract was passed over a Q-sepharose column equilibrated with 50 mM Tris-HCl pH 8.5, 1 mM EDTA, and 1 mM DTT. Bound Ets-1(29-138) was eluted with a gradient of NaCl. Ets-1(29-138) was dialyzed in 20 mM citrate buffer, pH 5.3, 1 mM EDTA, 10% glycerol, 150 mM KCl, and 1 mM DTT and was purified on a 16/60 Superdex-75 size exclusion column in the same buffer. Yields ranged from 40-50 mg protein/liter of culture. Protein was stored at -80°C at 3.6 ml (10 mg) aliquots.

Construction of affinity columns

Proteins were dialyzed in coupling buffer (25 mM HEPES-KOH pH, 7.5, 10% glycerol, 50 mM KCl, and 1 mM DTT). A 12 ml slurry (~10 mg of gel) of Affi-Gel 10 matrix (BioRad) equilibrated in 25 mM HEPES-KOH, pH 7.5, 10% glycerol, and 50 mM KCl was mixed with 70 mg of Ets-1(29-138) with agitation overnight at 4°C. Ets-1(29-138) was covalently bound to Affi-Gel 10 by amine-coupling. The amount of unbound Ets-1(29-138) was determined by measuring the absorbance at A_{280} of the supernatant. The starting amount of Ets-1(29-138) minus unbound Ets-1(29-138) was used to determine the coupling efficiency, which was 5-6 mg Ets-1(29-138)/ml of Affi-Gel 10 slurry. A control column was constructed with bovine serum albumin (BSA), which had a coupling efficiency of 3-6 mg BSA/ml of Affi-Gel 10. The affinity matrices were packed into HR10/16 C columns (Amersham-Pharmacia). Both the Ets-1(29-138) and BSA affinity columns contained approximately 30 mg of ligand and were approximately 5 ml in volume.
Preparation of calf thymus nuclear extract

Calf thymus were obtained from Pelfreeze. Two thymus were thawed in Buffer A (15 mM Tris-HCl, pH 7.9, 120 mM KCl, 15 mM NaCl, 2 mM EDTA, 250 mM sucrose) for 5 min. Thymus was defatted and cut into approximately 1 cm³ pieces. Pieces were mixed with ice cold Buffer B (10 mM Tris-HCl, pH 7.9, 25 mM KCl, 1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol, 2 M sucrose, 1 mM DTT, 2.5 mM benzamidine, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) at a ratio of 2 ml/g of tissue. Tissue was homogenized for 20 sec at high speed in an Oster commercial blender. Homogenate was filtered successively through 1, 2, and 3 layers of cheesecloth. Filtrate was centrifuged (9000 rpm, 30 min, JA-10 rotor) and supernatant was removed. The resulting nuclear pellet was resuspended in Buffer C (10 mM Tris-HCl, pH 7.9, 100 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 0.15 mM spermine, 0.5 mM spermidine, 10% glycerol, 1 mM DTT, 2.5 mM benzamidine, 0.5 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml pepstatin) to a concentration of approximately 3.5 x 10⁸ nuclei/ml of buffer (assuming 2.5 x 10⁸ nuclei per gram of thymus tissue). Ammonium sulfate was added to a final concentration of 0.4 M. Nuclei were lysed by rocking for 30 min at 4°C. Nuclear lysate was collected by ultracentrifugation at 40,000 rpm for 2 hr. Recovered lysate was passed through cheesecloth. Nuclear proteins were precipitated by adding 0.33 g of crushed ammonium sulfate per ml of extract followed by gentle stirring for 30 min. Precipitate was collected by centrifugation (30 min, 10,000 rpm, 4°C, JA-20 rotor followed by 30 min, 40,000 rpm) and was redissolved in 25 mM HEPES-KOH, pH 7.5, 50 mM KCl, 2 mM EDTA, 10% glycerol, 0.5 mM PMSF, and 1 mM DTT. The redissolved protein was dialyzed against the same buffer.
and undissolved protein was collected by centrifugation (30 min, 10,000 rpm, 4°C, JA-20 rotor). The concentration of soluble protein was determined by Bradford assay using BSA as a standard. Proteins were frozen in a dry ice/ethanol bath and stored at −80°C in 5 ml (5mg/ml) aliquots. The yield ranged from ~24–66 mg nuclear protein/100 g thymus (1 thymus~150 g).

**Affinity chromatography**

The affinity columns were equilibrated in 25 mM HEPES-KOH, pH 7.5, 10% glycerol, 1 mM EDTA, 50 mM KCl, and 1 mM DTT. Approximately 30 mg of calf thymus nuclear extract was passed over the affinity column at 0.4 ml/min. Columns were washed with ~10 column volumes of buffer containing 50 mM KCl to remove weakly binding proteins. Proteins were eluted with a 10 column volume gradient between 50 and 500 mM KCl. The column was then washed with 5 column volumes at 1 M KCl. Eluted proteins were collected in fractions, pooled, separated by SDS-PAGE, and detected by silver stain or staining with Coomassie Blue. Columns were used once and were stored at 4°C.

**Immunoblotting**

Anti-ERK2 rabbit affinity-purified polyclonal antibody was obtained from Santa Cruz Biotechnology. The UBC9 antibody was obtained from Transduction Laboratories. Standard procedures for immunoblotting were followed.

**Results**

To identify protein partners for the PNT domain and MAPK site biochemically, an affinity chromatography experiment was performed. Several materials were
generated for the experiment. First, an Ets-1(29-138) affinity column was constructed. Ets-1(29-138) was overexpressed in bacteria, purified by conventional chromatography (Fig. 4.1), and then covalently linked to Affi-Gel 10 resin by amine-coupling. The final matrix had a yield of approximately 5-6 mg Ets-1(29-138)/ml resin. Second, a calf thymus nuclear extract was prepared as the source of potential binding partners. Ets-1 is a nuclear protein expressed in T cells, which reside in the thymus; thus, the nuclei of this organ should contain interacting partners for Ets-1. In addition, large amounts of nuclear protein (~100 mg) can be obtained from a preparation of extract, which facilitates detection and identification of interacting proteins. Last, a column was constructed with bovine serum albumin (BSA). Because BSA and Ets-1(29-138) have similar calculated isoelectric points, the BSA column serves as a control for nonspecific electrostatic interactions.

To bind and retrieve interacting proteins, an identical amount of calf thymus nuclear extract was passed over the Ets-1(29-138) and the control BSA affinity columns. Binding was allowed to occur under low salt conditions (50 mM KCl) that favor the formation of protein interactions. To elute interacting proteins, electrostatic interactions were disrupted by increasing the concentration of KCl. Fractions were collected, pooled, concentrated, and subjected to SDS-PAGE and Coomassie Blue staining.

Multiple proteins bound to the Ets-1(29-138) column and eluted under low-salt conditions (100-200 mM KCl) (Fig. 4.2). Approximately 17 discrete, intensely staining bands were detected that ranged in estimated molecular mass from 10 to 170 kDa.
Figure 4.1 Overexpression of Ets-1(29-138). A brief description of the purification procedure is depicted on the left. The fragment was electrophoresed on a SDS-polyacrylamide gel and stained with Coomassie Blue (right).
Figure 4.2 Ets-1(29-138) affinity chromatography. (Left) Schematic of the purification procedure. (Right) Proteins that eluted from an Ets-1(29-138) and BSA affinity column are shown electrophoresed on a SDS-polyacrylamide gel that was stained with Coomassie Blue. Molecular masses are noted on the left. Prominently stained bands eluting from the Ets-1(29-138) column are highlighted on the right.
These proteins did not elute from the control column constructed with BSA, suggesting that these proteins bound due to the presence of the Ets-1(29-138) ligand.

A candidate approach was used to identify two of the interacting proteins. It was noted that the bands electrophoresing near the 43 kDa molecular mass marker were of approximately the same molecular mass as the MAPKs ERK1 and ERK2 (44 and 42 kDa, respectively) that are known to phosphorylate Ets-1 at the MAPK site (T38) (Yang et al., 1996). To determine if one of the major bands was either of these kinases, an immunoblot was performed with an antibody that recognizes an epitope found in both ERK1 and ERK2 (referred to as ERK1/2). In the immunoblot, the antibody recognized a protein of the same molecular mass expected for the kinases (Fig. 4.3). A quantitative western demonstrated that approximately equivalent amounts of ERK1/2 were recognized by the antibody as were estimated to be present in the major 42 kDa Coomassie stained band (data not shown). This suggests that the intensely Coomassie stained band of approximately 42 kDa was ERK1 and/or ERK2.

ERK1/2 can exist in two states: an unphosphorylated, inactive state, and a dual-phosphorylated active state. Dual-phosphorylation is also required for nuclear import of ERK1/2. The ERK1/2 recovered in the affinity chromatography experiment was unphosphorylated as determined by an immunoblot using an antibody specific for dual-phosphorylated ERK1/2 and an in vitro kinase reaction (data not shown). It was expected that ERK1/2 would be the phosphorylated form, as it was derived from a nuclear extract. It is possible that nuclear ERK1/2 was dephosphorylated by a phosphatase during purification of the calf thymus nuclear extract.
Figure 4.3 ERK1/2 interacts with Ets-1(29-138). An immunoblot was performed on fractions eluting with 100-200 mM KCl from the Ets-1(29-138) column. A band of the anticipated molecular mass for ERK1/2 was recognized by an anti-ERK1/2 antibody.
Another prominent protein that bound the Ets-1(29-138) column had an apparent molecular mass of 18 kDa (Fig. 4.2). This is the molecular mass of the SUMO conjugating enzyme UBC9, which had been reported to interact with an N-terminal fragment of Ets-1 in a yeast two-hybrid interaction assay (Hahn et al., 1997). An immunoblot confirmed that an 18 kDa protein that bound the affinity column was UBC9 (Fig. 4.4). This interaction was not further characterized.

The affinity chromatography experiment was repeated several times. The binding of proteins of approximately 42 kDa and 18 kDa was reproducible in three experiments. However, the banding pattern reported in Figure 4.2 was not consistently observed (data not shown). It is possible that protein degradation in the calf thymus nuclear extract (H. Wang, personal communication) affected the reproducibility of the other bands.

**Discussion**

There are many protein-protein interaction assays that can be used to screen or select for interacting proteins. An affinity chromatography experiment was performed here to take advantage of the ability of the Ets-1(29-138) fragment to be highly expressed in bacteria and easily purified by conventional chromatography. Identification of ERK1/2 and UBC9, previously reported partners for Ets-1, validated this approach as an effective means of retrieving Ets-1 partners.

**ERK1/2 interaction with Ets-1(29-138)**

The kinases ERK1/2 eluted from the affinity column under low salt conditions (100-200 mM KCl). This elution profile suggested that the affinity of ERK1/2 for
Figure 4.4 UBC9 interacts with Ets-1(29-138). Immunoblot with anti-UBC9 of fractions eluting from an Ets-1(29-138) affinity column under low salt conditions. A human endothelial lysate was used as a positive control.
Ets-1(29-138) is relatively weak. This is not surprising for an enzyme-substrate interaction. Also, elution of ERK1/2 due to increasing salt concentrations suggests that the interaction of ERK1/2 with the column was mediated in part by electrostatic interactions.

Only a small amount of the total ERK1/2 (<1%) in the calf thymus nuclear extract that was passed over the affinity column bound Ets-1(29-138) (data not shown). There may be several reasons for this observation. First, only a subpopulation of ERK1/2 from the calf thymus may have been competent for binding Ets-1(29-138). For instance, ERK1/2 that did not bind may have been complexed with other proteins. Second, only a fraction of the Ets-1(29-138) may have been coupled to the Affi-Gel 10 matrix in an orientation that allows binding to ERK1/2. There are 12 lysine and arginine residues on the surface of Ets-1(29-138) that could have been used to couple to Affi-Gel 10. Thus, many of the Ets-1(29-138) molecules may have been positioned in orientations unavailable for binding ERKs.

There are several modes by which ERK1/2 may have interacted with the affinity column. First, the interaction may have been mediated by the MAPK phosphoacceptor of the Ets-1 fragment, which lacks secondary structural elements and is highly flexible. This proposal is consistent with the idea that ERK1/2 must at least transiently interact with the MAPK phosphoacceptor during the phosphorylation reaction. However, due to its simple sequence, the phosphoacceptor might not be expected to form a stable interaction with the kinase. Second, ERK1/2 may have interacted with Ets-1(29-138) through an intermediate protein. Consistent with this hypothesis is the observation that a number of other proteins co-eluted with ERK1/2 in the affinity chromatography
experiment. However, there is no precedent in the literature for proteins mediating interactions between MAPKs and substrates. Third, it is possible that ERK1/2 interacted with the Ets-1 fragment distal to the phosphoacceptor. Such interaction sites are termed docking sites and have been identified in a number of transcription factors as well as other signaling proteins (Sharrocks et al., 2000). The hypothesis that the Ets-1 PNT domain contains an ERK2 docking site is investigated in Chapter 5.

Besides ERK1 and ERK2, there are other MAPKs in mammalian cells, including the p38 MAPKs, JNKs, and ERK5. To date, however, only ERK1, ERK2, and an isoform of JNK have been reported to phosphorylate T38 and T72 in Ets-1 and Ets-2, respectively (Yang et al., 1996; Smith et al., 2000). It was not tested whether JNKs or other kinases from the calf thymus nuclear extract interacted with the Ets-1(29-138) affinity column.

**UBC9 interaction with Ets-1(29-138)**

UBC9 interacted with the Ets-1(29-138) affinity column and eluted under low salt conditions. It was reported that UBC9 interacted with Ets-1(1-130), which contains part of the PNT domain, in a yeast two-hybrid interaction assay (Hahn et al., 1997). It was not clear from that report whether the PNT domain was involved in the interaction. The PNT domain of TEL, however, can interact with and be modified by UBC9 (Chakrabarti et al., 1999; Chakrabarti et al., 2000). Strikingly, UBC9 can also interact with other ets proteins that lack a PNT domain, including Elf-1, PU.1, and Net.

UBC9 conjugates small ubiquitin-related modifier (SUMO) to proteins. Unlike ubiquitination, which targets proteins for degradation, SUMO conjugation appears to play a role in modulating protein-protein interactions and subcellular localization.
(Melchior, 2000). It was also reported that co-expression of UBC9 and Ets-1 in HeLa cells resulted in increases in transactivation of reporters by Ets-1 (Hahn et al., 1997), despite the fact that SUMO modification of Ets-1 was not detected. Given the reports of SUMO function, it is predicted that modification of Ets-1 by UBC9 should target Ets-1 to subnuclear structures termed PODs (Doucas, 2000). More investigation is required to understand the nature and function of the UBC9 interaction with Ets-1.

**Other potential partners of Ets-1(29-138)**

There may be other protein partners for the Ets-1 PNT domain and MAPK site. This is supported by the observation that approximately 15 other proteins bound the Ets-1(29-138) affinity column in the affinity chromatography experiment (Fig. 4.2). While each protein may have bound Ets-1(29-138) independently, it is possible that several of these proteins bound as a complex. Candidates for protein complexes that could interact with Ets-1 include histone acetylase and deactylase complexes and complexes that contain the basal transcriptional machinery. Identification of the proteins that bound the affinity column would facilitate a broader understanding of the role of Ets-1 in regulating transcriptional initiation of specific genes.

**References**


CHAPTER 5

AN ERK2 DOCKING SITE IN THE POINTED DOMAIN
DISTINGUISHES A SUBSET OF ets TRANSCRIPTION FACTORS
Abstract

The ets transcription factors perform distinct biological functions despite conserving a highly similar DNA binding domain. One distinguishing property of a subset of ets proteins is a conserved region of 80 amino acids termed the Pointed (PNT) domain. Using enzyme kinetics we determined that the Ets-1 PNT domain contains an ERK2 docking site. The docking site enhances the efficiency of phosphorylation of a mitogen-activated protein kinase (MAPK) site N-terminal to the PNT domain. The site enhances ERK2 binding rather than catalysis. Three hydrophobic residues are involved in docking, and the previously determined NMR structure (Slupsky et al., PNAS, 95, 12129-12134, 1998) indicates that these residues are clustered on the surface of the Ets-1 PNT domain. The docking site function is conserved in the PNT domain of the highly related Ets-2 but not in the ets family member GABPα. Ablation of the docking site in Ets-1 and Ets-2 prevented Ras pathway mediated enhancement of the transactivation function of these proteins. This study provides structural insight into the function of a MAPK docking site and describes a unique activity for the PNT domain among a subset of ets family members.

Introduction

The ets gene family encodes evolutionarily related transcription factors that regulate multiple biological functions. Members of this family conserve an 85 amino acid DNA binding ETS domain that binds the core DNA sequence 5' -GGA(A/T)-3' (Graves and Petersen, 1998; Sharrocks, 2001). Ets genes are found in all metazoans studied to date and number 8 in Drosophila (Hsu and Schulz, 2000), 10 in C. elegans (Hart et al., 2000), and 25 in humans. Strikingly, distinct ets proteins can regulate
diverse biological processes despite having the ability to bind highly similar DNA sequences. For instance, gene targeting studies in mice indicate that different ets proteins play critical roles in varied physiological processes such as hematopoiesis, apoptosis, and regulation of the extracellular matrix (Bartel et al., 2000). In addition, different ets genes are responsible for regulating embryogenesis and neural development in Drosophila (Hsu and Schulz, 2000). This diversity of function raises the question of how specificity is generated among members of this gene family.

To regulate the transcription of unique genes, individual ets proteins have evolved different molecular features. One feature conserved in approximately 40% of ets family members is the 80 amino acid Pointed (PNT) domain. Surprisingly, this domain can perform distinct functions among ets proteins. For instance, the PNT domain of the ets family member TEL can serve as a transcriptional repression module (Fenrick et al., 1999; Wang and Hiebert, 2001). The repression activity may be related to the ability of the PNT domain to form helical polymers (Kim et al., 2001). The TEL PNT domain is also fused to heterologous proteins as a result of translocations associated with leukemia (Golub et al., 1994; Golub et al., 1995; Golub et al., 1996; Lacronique et al., 1997). Oligomerization of the PNT domain is hypothesized to play an important role in the genesis of the associated leukemia. In contrast, the Ets-1 PNT domain enhances the activity of the Ets-1 transactivation domain but does not function in transcriptional repression (Schneikert et al., 1992). Also, unlike the TEL PNT domain, the Ets-1 PNT domain is monomeric (Slupsky et al., 1998). Biochemical reasons for differences in function are suggested by structural studies that indicate that the PNT domains of these two proteins have similar folds but divergent surface
chemistry. Due to the lack of conservation of surface residues, PNT domains of different ets proteins may interact with distinct protein partners, thereby establishing unique biological functions within the family.

The specificity of ets protein function also is enhanced by signal transduction pathways (Yordy and Muise-Helmericks, 2000). For instance, the RAS/RAF/MEK/ERK pathway modulates the activities of vertebrate Ets-1 and Ets-2 and their apparent ortholog in Drosophila, Pnt-P2. These proteins are phosphorylated at a conserved mitogen-activated protein kinase (MAPK) phosphoacceptor site N-terminal to the PNT domain (Brunner et al., 1994; O'Neill et al., 1994; Yang et al., 1996). The MAPKs ERK1 and ERK2 phosphorylate Ets-1 and Ets-2 at this site, and the highly related Drosophila MAPK Rolled phosphorylates Pnt-P2. Phosphorylation of this site results in enhancement of the transactivation activity of these proteins by an undetermined mechanism (Brunner et al., 1994; O'Neill et al., 1994; Yang et al., 1996). The biological relevance of this phosphoacceptor is firmly established by genetic studies in Drosophila that demonstrate that MAPK phosphorylation of Pnt-P2 is required for R7 photoreceptor development (Brunner et al., 1994; O'Neill et al., 1994).

This report demonstrates that the PNT domain regulates Ets-1 and Ets-2 phosphorylation by serving as an ERK2 docking site. Docking sites are defined as short sequence motifs that lie distal to the phosphoacceptor in the linear amino acid sequence and increase the efficiency of substrate phosphorylation (Holland and Cooper, 1999; Sharrocks et al., 2000). In addition, these motifs can affect the accuracy and specificity of MAPK phosphorylation. Docking sites are thought to function by interacting with MAPKs outside the catalytic site, thereby increasing the concentration of the
phosphoacceptor near the enzyme. A number of classes of proteins contain MAPK docking sites, including kinases, phosphatases, scaffold proteins, and transcription factors.

Despite numerous examples, little is known about the structural details of docking sites. To date, docking sites in transcription factors have been identified exclusively by deletion and site-directed mutagenesis of residues that are not defined by secondary or tertiary structural information. Lacking a structural context, these mutagenesis experiments could not identify residues that define the docking interfaces. Additionally, although docking sites often lie distant to the phosphoacceptor in the linear amino acid sequence, the relative positions of these elements in three-dimensional space is not known.

This report provides insights into the structural features of transcription factor docking sites as well as the specificity of ets protein function. The previously determined NMR structure of the PNT domain allowed us to map a hydrophobic surface on the Ets-1 PNT domain required for efficient phosphorylation by ERK2. Kinetic data indicate that the residues in this surface mediate a binding event rather than an alteration of catalysis. Enzyme kinetics also indicate that the docking site is conserved in the PNT domain of Ets-2 but not in the ets family member GABPα. Functionally, the docking site is required for Ras pathway mediated enhancement of the transactivation activity of Ets-1 and Ets-2. In sum, this study reveals the structural context of a MAPK docking site in a transcription factor and highlights ERK2 docking as a distinguishing feature of a subset of PNT domains of ets family members.
Results

ERK1/2 bind an Ets-1(29-138) affinity column

The PNT domain is hypothesized to be a protein-protein interaction motif. To identify protein partners, an affinity chromatography experiment was performed using Ets-1(29-138) as a ligand, which includes the PNT domain (residues 54-132) and MAPK phosphoacceptor site (T38). A calf thymus nuclear extract was used as a source of potential partner proteins due to the abundance of Ets-1 in T lymphocytes. In the chromatography experiment, several proteins from the extract bound the Ets-1(29-138) column, and one protein was identified by immunoblotting to be the MAPK ERK1 and/or ERK2 (data not shown). The ERKs may have interacted with Ets-1 solely through the MAPK phosphoacceptor; however, we speculated that a docking site distal to the MAPK phosphorylation site also mediated the interaction.

The Ets-1 PNT domain contains an ERK2 docking site

To localize a potential ERK2 docking site in Ets-1, full-length Ets-1 and two fragments of Ets-1 were generated for kinase assays (Fig. 5.1). Full-length Ets-1 contains the MAPK phosphorylation site (T38), PNT domain, transactivation domain, and DNA binding ETS domain (Fig. 5.1). The two fragments were designed based on NMR solution structure data (Chapter 2; Slupsky et al., 1998). The Ets-1(1-138) fragment retains the MAPK phosphorylation site that lies within disordered sequences and the globular five α-helix PNT domain (Fig. 5.1, 5.2, 5.3). The Ets-1(1-52) fragment retains only the MAPK phosphorylation site (Fig. 5.1) and presumably lacks secondary structural elements. Each fragment also contains a histidine tag to facilitate purification.
Figure 5.1 Schematic of ets fragments. MAPK phosphorylation site (T38) (asterisk); box shading demarcates PNT domains (gray, hatched), ETS domain (black), and histidine tag (stippled). The transactivation domain (TAD) of Ets-1 lies between the PNT domain and ETS domain.
**Figure 5.2** Sequence lineup of the PNT domain. Position of docking residues in the secondary structure of Ets-1(1-138). Rectangles indicate α-helices and the asterisk indicates the position of the MAPK phosphorylation site (T38) within the flexible N-terminal region of Ets-1 (Slupsky et al., 1998) (top). Sequence lineup (bottom) of helix H4 and the loop between helices H4 and H5 of the PNT domains of *ets* family members as well as *Drosophila* Mae, which lacks an ETS domain. Residues conserved in >50% of proteins are shaded in black; conservative substitutions found in 50% of proteins are shaded in gray. M, murine; H, human; D, *Drosophila*; C, *C. elegans*. Numbering is for murine Ets-1. Arrowheads mark residues involved in ERK2 docking in Ets-1 and Ets-2 (Table 5.1, 5.2)
Figure 5.3 Structure of the ERK2 docking site. Positions of docking residues in the NMR solution structure of Ets-1(29-138) (Chapter 2; Slupsky et al., 1998). Ribbon display (left) and surface rendering (right) show α-helices H1-H5. Helix H4, H5, and the connecting loop are shaded in dark gray (top). Mutagenized residues are highlighted: residues that gave negligible increases in $K_m$ when mutated (yellow); residues that gave four-fold or higher increases in $K_m$ when mutated (green). The disordered N-terminal region containing the phosphoacceptor (T38) (red) is shown in ribbon display and in different orientations to highlight its flexibility (left and right). The PNT domain also shows strong structural similarity to the SAM domain in helices H2-H5 (Stapleton et al., 1999). The figure was generated using RasMol and WebLab ViewerPro 4.0 (Accelrys Inc.).
Kinase assays were performed in vitro under steady-state conditions with [γ-32P]ATP and the initial rates of phosphorylation were fit to the Michaelis-Menten equation (Fig. 5.4A). The parameter $K_m$, the apparent equilibrium dissociation constant of a kinase and its substrate, was used to compare the ability of ERK2 to bind ets fragments. The $K_m$ of the Ets-1(1-138) fragment was nearly identical to that of full-length Ets-1 (6.8 ± 1.8 μM and 5.1 ± 1.2 μM, respectively; Fig. 5.4B). The fragment was phosphorylated on T38, as evidenced by the lack of phosphorylation of Ets-1(1-138;T38A) under assay conditions (data not shown). Additionally, the histidine tag did not affect the $K_m$ value (data not shown). These data suggest that the first 138 amino acids of Ets-1 are sufficient to achieve full binding activity with ERK2.

In contrast, Ets-1(1-52), which lacks the PNT domain and known secondary structural elements, had a $K_m$ approximately 30-fold higher (190 ± 35 μM) than that of full-length Ets-1 and Ets-1(1-138) (Fig. 5.4B). The $K_m$ of this fragment is on the order of magnitude of previously characterized peptide substrates of ERK2 (~300-500 μM; Gonzalez et al., 1991; Robinson et al., 1996). The $k_{cat}$ value showed only a slight two-fold decrease, suggesting that removal of the PNT domain did not drastically affect enzyme catalysis. These data argue that the PNT domain contains an ERK2 docking site that enhances the binding affinity of the enzyme-substrate interaction.

L114, L116, and F120 form a potential docking interface

To define the docking site, the NMR solution structure of Ets-1(29-138) (Chapter 2; Slupsky et al., 1998) was used to select surface residues of the PNT domain of Ets-1(1-138) for site-directed mutagenesis. The residues that were initially targeted were an LXL sequence (L114, L116) and a basic residue (K110) on the surface of helix
Figure 5.4 The Ets-1 PNT domain contains an ERK2 docking site.

(A) Phosphorylation of Ets-1 by ERK2 follows Michaelis-Menten kinetics. Ets-1(1-138) was phosphorylated by ERK2 with \( [\gamma^{32}\text{P}] \) ATP under steady-state conditions as described in the Materials and methods. Phosphorimage of a 15% SDS-polyacrylamide gel (left) displays reaction products. Initial reaction velocities, \( v \), were divided by the ERK2 concentration, \([E]_0\). Duplicate \( v/[E]_0 \) values from a single experiment were averaged and are shown fit to the Michaelis-Menten equation \( v/[E]_0 = k_{\text{cat}}[S]/(K_m + [S]) \), where \([S]\) is the ets protein concentration (right). Error bars indicate the range.

(B) Kinetic parameters of Ets-1 and fragments phosphorylated by ERK2. Mean values for \( K_m \) and \( k_{\text{cat}} \) are reported ± S.E. and were determined as described in the Materials and methods and (A) using data from two (Ets-1), five (Ets-1(1-138)), and three (Ets-1(1-52)) independent experiments. \( k_{\text{cat}}/K_m \) was determined by dividing individual values.

<table>
<thead>
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<th>Protein</th>
<th>( K_m ) (( \mu \text{M} ))</th>
<th>( k_{\text{cat}} ) (min(^{-1}))</th>
<th>( k_{\text{cat}}/K_m ) (( \mu \text{M}^{-1}\text{min}^{-1} ))</th>
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<td>Ets-1</td>
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<td>180</td>
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<td>1000 ± 86</td>
<td>150</td>
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<tr>
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<td>550 ± 37</td>
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H4 (Fig. 5.2, 5.3). These residues are in a sequence pattern described for other MAPK docking sites (Holland and Cooper, 1999; Sharrocks et al., 2000). Mutation of L114 and L116 to alanines or arginines resulted in a modest 4-fold increase in $K_m$, whereas mutation of K110 to alanine did not result in a significant increase in $K_m$ (Table 5.1). These results suggest that the leucines, but not K110, play a role in ERK2 binding. However, the binding activity of these mutants was stronger than that of Ets-1(1-52) (Fig. 5.4B), which lacks the PNT domain, suggesting that other residues of the PNT domain also contribute to ERK2 binding. Mutation to alanine of the residues C112 and E115 (Fig. 5.2), which lie near L114 and L116 on the surface of the PNT domain (Fig. 5.3), did not alter the $K_m$ (Table 5.1). In contrast, mutation of the surface residue F120 (Fig. 5.2, 5.3) to alanine resulted in a striking 29-fold increase in $K_m$ (Table 5.1). This mutation, when combined with the L114R and L116R mutations, resulted in a fragment with a $K_m$ 40-fold higher than that of Ets-1(1-138) and similar to that of the fragment deleting the PNT domain, Ets-1(1-52) (Table 5.1). These data indicate that L114, L116, and in particular, F120, play an important role in Ets-1 docking with ERK2. The role of the phenylalanine also indicates that the site is distinct from other LXL containing docking sites.

The location of L114, L116, and F120 on the surface of the Ets-1 PNT domain (Fig. 5.3) suggests that these residues form an interface that contacts ERK2. Using partial trypsin proteolysis, we addressed an alternative possibility that single amino acid substitutions could affect docking by disrupting the structure of the PNT domain. Potential trypsin proteolysis sites exist throughout Ets-1(1-138) (Fig. 5.5A). However, the wild-type PNT domain was refractory to cleavage (Fig. 5.5A), presumably due to
<table>
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<th>$K_m^a$ (µM)</th>
<th>Fold increase in $K_m^b$</th>
<th>$k_{cat}^a$ (min$^{-1}$)</th>
<th>$k_{cat}/K_m^c$ (µM$^{-1}$min$^{-1}$)</th>
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</thead>
<tbody>
<tr>
<td>Ets-1(1-138)</td>
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<td>-</td>
<td>1000 ± 86</td>
</tr>
<tr>
<td>C112A</td>
<td>(2)</td>
<td>7.3 ± 1.1</td>
<td>1.1 ± 0.33</td>
<td>970 ± 47</td>
</tr>
<tr>
<td>E115A</td>
<td>(2)</td>
<td>8.6 ± 2.6</td>
<td>1.2 ± 0.48</td>
<td>1100 ± 120</td>
</tr>
<tr>
<td>K110A</td>
<td>(2)</td>
<td>11 ± 1.6</td>
<td>1.6 ± 0.48</td>
<td>1100 ± 60</td>
</tr>
<tr>
<td>L114A;L116A</td>
<td>(3)</td>
<td>27 ± 12</td>
<td>4.0 ± 2.1</td>
<td>1100 ± 150</td>
</tr>
<tr>
<td>L114R;L116R</td>
<td>(3)</td>
<td>29 ± 8</td>
<td>4.3 ± 1.6</td>
<td>1200 ± 92</td>
</tr>
<tr>
<td>F120A</td>
<td>(4)</td>
<td>200 ± 24</td>
<td>29 ± 8.4</td>
<td>860 ± 43</td>
</tr>
<tr>
<td>L114R;L116R:F120A</td>
<td>(2)</td>
<td>270 ± 63</td>
<td>40 ± 14</td>
<td>850 ± 64</td>
</tr>
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</table>

$^a$Value ± S.E. determined by nonlinear least squares fit to the Michaelis-Menten equation.

$^b$Relative to the $K_m$ of Ets-1(1-138).

$^c$Determined by dividing individual values.

$^d$Number of independent experiments performed in duplicate.

$^e$Similar value obtained by Wass and Dalby (2001).
**Figure 5.5** Trypsin probing of ets protein structure. (A) Schematic of Ets-1(1-138) depicting potential trypsin cleavage sites. Histidine tag (stippled), PNT domain with α-helices (gray with white boxes), potential trypsin cleavage sites (vertical lines). Ets-1(1-138;L114R;L116R;F120A) has two additional potential cleavage sites (L114R and L116R, longer lines). Arrowheads indicate residues, clustered as T1 and T2, that were cleaved by partial proteolysis of Ets-1(1-138) as determined by N-terminal sequencing. (B) Partial trypsin digest of Ets-1(1-138) (top) and Ets-1(1-138;L114R;L116R;F120A) (bottom). Proteins were incubated with trypsin for indicated times, then electrophoresed on an 18% SDS-polyacrylamide gel. Digital image shows Coomassie Blue stained gels with size standards noted (kDa). Clusters of cleavage sites (T1 and T2) in (A) are the proposed N-termini of the indicated protein bands.
the highly folded structure of the domain (Chapter 2; Slupsky et al., 1998) (Fig. 5.3). Thus, mutations that severely disrupt the structure of the PNT domain are predicted to alter the pattern of protease sensitivity. The kinetics and pattern of partial trypsin proteolysis of Ets-1(1-138; L114R; L116R; F120A) were nearly identical to those of Ets-1(1-138) (Fig. 5.5B), suggesting that the mutations did not disrupt the fold or flexibility of the PNT domain. These data, combined with the NMR-based structure and kinase assays, suggest that L114, L116, and F120 comprise an interface that docks with ERK2.

**The docking site is conserved in the PNT domain of Ets-2**

Ets-1 and Ets-2 are closely related proteins that conserve both the MAPK phosphorylation site and the PNT domain. The amino acid sequences spanning these functional elements are 70% identical, suggesting that Ets-1 and Ets-2 have similar three-dimensional structures in these regions. Notably, the residues comprising the ERK2 docking site in Ets-1 are conserved in Ets-2 (L148, L150, and F154) (Fig. 5.2), predicting that the PNT domain of Ets-2 also contains an ERK2 docking site.

To test for a functional ERK2 docking site, kinase assays were performed with a histidine-tagged fragment of Ets-2 encompassing amino acids 1-172 that include the MAPK site (T72) and PNT domain (Fig. 5.1). The \( K_m \) and \( k_{cat}/K_m \) of this protein (Table 5.2) were similar to the values for Ets-1(1-138) (Table 5.1). Mutation of the proposed docking residues L148 and L150 to arginines resulted in a 5-fold increase in \( K_m \) (Table 5.2), akin to the effect of mutating L114 and L116 in Ets-1 (Table 5.1). Strikingly, mutation of the other proposed docking residue, F154, to alanine caused a 75-fold increase in \( K_m \) (Table 5.2), and combination of the L148R, L150R, and F154A
Table 5.2. Kinetic analysis of Ets-2(1-172)

<table>
<thead>
<tr>
<th>Protein [Ets-2(1-172)]</th>
<th>$K_m^a$ (µM)</th>
<th>Fold increase in $K_m^b$</th>
<th>$k_{cat}^a$ (min⁻¹)</th>
<th>$k_{cat}/K_m^c$ (µM⁻¹min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ets-2(1-172)</td>
<td>2.8 ± 0.41</td>
<td>-</td>
<td>420 ± 19</td>
<td>150</td>
</tr>
<tr>
<td>L148R;L150R</td>
<td>15 ± 3.1</td>
<td>5.4 ± 1.4</td>
<td>720 ± 46</td>
<td>48</td>
</tr>
<tr>
<td>F154A</td>
<td>210 ± 37</td>
<td>75 ± 17</td>
<td>560 ± 38</td>
<td>2.7</td>
</tr>
<tr>
<td>L148R;L150R;F154A</td>
<td>270 ± 51</td>
<td>96 ± 23</td>
<td>530 ± 40</td>
<td>2.0</td>
</tr>
</tbody>
</table>

$^a$Value ± S.E. determined by nonlinear least squares fit of data from two independent experiments performed in duplicate to the Michaelis-Menten equation.

$^b$Relative to the $K_m$ of Ets-2(1-172).

$^c$Determined by dividing individual values.
mutations resulted in a 96-fold increase in \( K_m \) (Table 5.2). These values were accompanied by minimal changes in \( k_{cat} \), indicating disruption of a binding event. Indistinguishable trypsin proteolysis patterns were observed for Ets-2(1-172) and Ets-2(1-172;L148R;L150R;F154A) (data not shown), suggesting that the mutations do not significantly alter the structure of the Ets-2 PNT domain. These data demonstrate that the ERK2 docking site is conserved in Ets-2.

The *Drosophila* protein Pnt-P2 is encoded by a gene that is an apparent ortholog of the genes encoding Ets-1 and Ets-2. Pnt-P2 conserves the Ras/MAPK targeted phosphoacceptor found N-terminal to the PNT domain in Ets-1 and Ets-2 (Brunner et al., 1994; O’Neill et al., 1994). Additionally, the PNT domain of Pnt-P2 contains sequence closely resembling the ERK2 docking site of Ets-1 and Ets-2 (L228, I230, and F234; Fig. 5.2). It is likely this sequence functions as a docking site for the *Drosophila* MAPK related to ERK2, Rolled.

The docking site is not conserved in the PNT domain of GABP\( \alpha \)

The juxtaposition of the MAPK phosphorylation site and the PNT domain is not conserved in *ets* proteins other than vertebrate Ets-1 and Ets-2 and *Drosophila* Pnt-P2. This suggests that the other PNT domains may not serve as ERK2 docking sites. Consistent with this hypothesis, the residues involved in docking in Ets-1 and Ets-2 are not well-conserved in other PNT domains (Fig. 5.2). In GABP\( \alpha \), for instance, the two leucines of the docking site in Ets-1 are replaced by a phenylalanine and an arginine, and the loop between helix H4 and H5 is shortened and lacks the phenylalanine (Fig. 5.2). Thus, although GABP\( \alpha \) is phosphorylated by MAPKs at other sites (Fromm and
Burden, 2001), we predicted that the PNT domain of GABPα would not contain an ERK2 docking site.

To test this prediction, a chimeric protein was generated by fusing the N-terminal extension of Ets-1 (residues 1-50) to the PNT domain of GABPα (Fig. 5.1). In kinetic assays with ERK2, the Ets-1/GABPα chimera had a 56-fold higher $K_m$ (380 ± 81 μM) than that of Ets-1(1-138) (Table 5.1). Furthermore, $k_{cat}/K_m$ for the chimera (2.1 μM⁻¹min⁻¹) was similar to that of Ets-1(1-52) and Ets-1(1-138;L114R;L116R;F120A) (Fig. 5.4B, Table 5.1). These data indicate that the PNT domain of GABPα does not efficiently dock with ERK2 and supports the hypothesis that the LXLXXXF motif is critical for docking.

To more rigorously establish a docking role for the LXLXXXF sequence, the docking residues were introduced into the GABPα PNT domain and tested for ERK2 binding by kinetic analysis. Leucine residues were inserted at the appropriate positions in the predicted helix H4 of the GABPα PNT domain, and a phenylalanine was introduced in the loop between helix H4 and H5 (Fig. 5.2). A valine was also introduced immediately C-terminal of the phenylalanine to conserve the spacing of the loop (Fig. 5.2). Introduction of the docking residues resulted in a 2.4-fold lower $K_m$ (160 ± 35 μM) than that of the Ets-1/GABPα chimera with a wild-type GABPα PNT domain. We conclude that introduction of these residues generated a docking site, albeit compromised relative to Ets-1 and Ets-2. It is possible that the side chains of the introduced docking residues may not be oriented in the GABPα PNT domain in an optimal conformation for ERK2 interaction. Alternatively, additional residues may contribute to full docking site function in Ets-1 and Ets-2.
The docking site is required for Ras/MAPK pathway signaling

Phosphorylation of Ets-1 and Ets-2 in vivo by the RAS-RAF-MEK-ERK pathway stimulates transactivation of genes regulated by Ras responsive elements (RREs) (Yang et al., 1996). Enhancement of transactivation by Ets-1 and Ets-2 is reduced by ablating the MAPK phosphoacceptor site (Yang et al., 1996). Therefore, mutations in the docking site that decrease levels of phosphorylation are predicted to reduce Ras/MAPK pathway mediated enhancement of the transactivation activity of Ets-1 and Ets-2.

To test the in vivo function of the docking site, a transient expression reporter assay was established in NIH3T3 cells. The reporter was constructed by inserting the RRE from the promoter of the matrix metalloproteinase-9 gene, which has a composite ets/AP-1 binding site (Watabe et al., 1998), upstream of the firefly luciferase gene. Plasmids encoding full-length Ets-1, Ets-2, or the phosphoacceptor mutants Ets-1(T38A) and Ets-2(T72A), were introduced into cells with or without a plasmid encoding a constitutively active form of MEK1 (CA-MEK1) (Mansour et al., 1994). MEK1 specifically phosphorylates and activates ERK1 and ERK2, which are endogenous in NIH3T3 cells, but not other subfamilies of MAPKs (Chang and Karin, 2001). The results for the wild-type and phosphoacceptor mutants are consistent with previous studies (Fig. 5.6, 5.7) (Yang et al., 1996) that demonstrate cooperation between ets proteins and the Ras signaling pathway in the stimulation of transcription and the requirement of T38 and T72 of Ets-1 and Ets-2, respectively, for enhancement of transactivation.
Figure 5.6. The Ets-1 docking site functions in the Ras pathway. Reporter assay with Ets-1 docking site mutants. NIH3T3 cells were transfected with a firefly luciferase gene reporter under the control of a MMP-9 Ras responsive element (RRE) (2.5 µg) and the pRL-null internal control plasmid (0.5 µg). As indicated, a plasmid encoding a constitutively active form of MEK1 (CA-MEK1) (0.1 µg) and a plasmid encoding wild-type or mutant Ets-1 (0.1 µg) were cotransfected. Firefly luciferase activity was normalized to Renilla luciferase activity (relative luciferase activity, RLA). Bars depict the mean ± S.D. of a representative experiment with each assay performed in duplicate (Ets-1 and mutants thereof transfected without CA-MEK1 were performed once). The experiment was repeated four times with similar results. Inset shows protein expression levels as detected by immunoprecipitation from metabolically labeled, transfected cells: Lane 1, mock transfected; 2, FLAG-Ets-1; 3, FLAG-Ets-1(T38A); 4, FLAG-Ets-1(L114R;L116R); 5, FLAG-Ets-1(F120A); 6, FLAG-Ets-1(L114R;L116R;F120A).
**Figure 5.7** The Ets-2 docking site functions in the Ras pathway. Transfection as in Fig. 5.6 with plasmids encoding wild-type or mutant Ets-2 (0.1 μg). Inset shows protein expression levels as determined above; dots indicate background bands; arrowhead, ets protein: Lane 7, mock transfected; 8, FLAG-Ets-2; 9, FLAG-Ets-2(T72A); 10, FLAG-Ets-2(L148R,L150R); 11, FLAG-Ets-2(F154A); 12, FLAG-Ets-2(L148R,L150R,F154A).
To assess the role of the docking site in transactivation, constructs expressing docking site mutants of full-length Ets-1 or Ets-2 were used in the transient expression assay. Strikingly, Ets-1(L114R;L116R), Ets-1(F120A), and Ets-1(L114R;L116R;F120A) had abrogated MEK1 stimulation to the same degree as mutation of the phosphoacceptor site (Fig. 5.6). The Ets-2 docking site mutants differed slightly in that they showed a graded ability to transactivate the reporter (Fig. 5.7). The ability to transactivate was consistent with the graded pattern of increase in $K_m$ observed for proteins with the same mutations in kinase assays (Fig. 5.7, Table 5.2). A parallel set of experiments performed with FLAG-epitope tagged ets proteins displayed similar results except that both FLAG-tagged Ets-1 and Ets-2 docking site mutants showed a graded ability to transactivate the reporter (data not shown), similar to un-tagged Ets-2 mutants (Fig. 5.7). Controls showed that protein levels in transfected NIH3T3 cells were similar for wild-type and mutant ets proteins (Fig. 5.6, 5.7). The simplest interpretation of these data is that mutation of the docking site reduces phosphorylation of Ets-1 and Ets-2 at T38 or T72, respectively, resulting in lower levels of transactivation. We conclude that the docking site plays an important role in Ras pathway dependent enhancement of transactivation by Ets-1 and Ets-2.

**Discussion**

Structural and mechanistic insights into ERK2 substrate docking

This report identifies three hydrophobic residues in the PNT domain that enhance phosphorylation of Ets-1 by ERK2. Several lines of evidence suggest that these residues form a hydrophobic ERK2 interaction surface, despite residing approximately 80 amino acids C-terminal of the ERK2 phosphoacceptor site. First, in
phosphorylation assays with ERK2, mutation of L114, L116, and F120 caused a 40-fold increase in $K_m$. This increase was not accompanied by large changes in $k_{cat}$, indicating that the mutations disrupted ERK2 binding rather than catalysis. Second, in the NMR solution structure, L114, L116, and F120 are clustered on the surface of the Ets-1 PNT domain where they could simultaneously contact ERK2. Third, the triple point mutant and the PNT domain deletion mutant had similar $k_{cat}/K_m$ values, indicating an essential role for L114, L116, and F120 in ERK2 docking. Finally, Ras/MAPK mediated enhancement of the transactivation activity of Ets-1 was reduced by mutation of the hydrophobic residues, showing that the docking site functions in vivo within the context of the full-length protein. Docking sites exist in many other proteins; however, this hydrophobic surface of the Ets-1 PNT domain is the first kinase docking interface in a transcription factor to be defined by high-resolution structural data.

Using the available structures, we sought to model the interaction of dual-phosphorylated ERK2 (Canagarajah et al., 1997) with the phosphoacceptor and docking site of Ets-1 (Slupsky et al., 1998). Due to the highly flexible nature of the extension containing the phosphoacceptor in Ets-1, one specific interaction could not be modeled. Instead, we estimated the potential surfaces of ERK2 with which the Ets-1 docking site might interact assuming that the phosphoacceptor and the docking site simultaneously contact ERK2. To delimit the range of potential interaction surfaces for the docking site, the phosphoacceptor was tethered in the ERK2 catalytic site and the PNT domain was rotated around the surface of ERK2 (Fig. 5.8).

Because the Ets-1 docking site is hydrophobic, it is assumed to interact with a hydrophobic surface of ERK2. Several hydrophobic surfaces lie within the determined
Figure 5.8 Potential docking interfaces of ERK2.
Potential docking interfaces of ERK2 as determined by molecular modeling (area within dashed line) on two views of activated ERK2 structure (Canagarajah et al., 1997). Dual-phosphorylated ERK2 can dimerize with a $K_d$ of 7.5 nM (Khokhlatchev et al., 1998); however, monomeric ERK2 was used for modeling, because most ERK2 was estimated to be monomeric at the concentrations (0.1 nM) used in the kinase assays. Hydrophobic residues (Val, Ile, Leu, Trp, Phe, Ala, and Met) are shown in green. Surfaces involved in the catalytic site (1) and ERK2 dimer interface (2) are noted. The ED (TT) site (3), CD domain (4), and surface containing Y314 and Y315 (5) are implicated to dock with other proteins (Tanoue et al., 2000; Tanoue et al., 2001; Xu et al., 2001). The figure was generated using WebLab ViewerPro 4.0 (Accelrys Inc.).
range (Fig. 5.8). Interestingly, surfaces of ERK2 proposed to mediate interactions with other docking sites in phosphatases, upstream and downstream kinases, and the transcription factor Elk-1 do not lie within our estimated range (Fig. 5.8) (Tanoue et al., 2000; Tanoue et al., 2001; Xu et al., 2001). Because known sites are not within our modeled range, we propose that the Ets-1 PNT domain interacts with a surface that has not been previously described as a docking interface on ERK2.

Kinetics and thermodynamics of docking

Direct binding of the Ets-1 PNT domain to ERK2 was not detected in several protein interaction assays (data not shown). Despite having micromolar $K_m$ values, ERK2 interactions with Ets-1 that precede phosphorylation or occur in the absence of ATP may have high dissociation rate constants, making them difficult to detect by conventional interaction assays. However, the interaction with ERK2 was detected on the affinity column using Ets-1(29-138) as a ligand (data not shown). The high concentration of ligand on the column matrix may have favored detection of this binding event. We do not suspect that an auxiliary protein mediated the interaction because purified, recombinant ERK2 could also bind the column (data not shown).

Despite an inability to detect direct binding, kinetic assays indicate that the docking site plays an important role in the efficiency of phosphorylation. This observation is supported by examining the thermodynamic contribution of the docking site to binding ERK2 during the phosphorylation reaction. Calculated based on $k_{cat}/K_m$ values, the interaction of ERK2 with the Ets-1(1-138) fragment had a Gibbs standard free energy ($\Delta G^0$) of approximately $-7.1 \text{ kcal/mol}$. In contrast, the $\Delta G^0$ of the Ets-1 triple mutant interaction with ERK2 was approximately $-4.9 \text{ kcal/mol}$. Thus, the free
energy contribution to binding ($\Delta G^\circ$) of the docking site was approximately 2.2 kcal/mol, a significant portion (~30%) of the overall binding energy, illustrating the importance of the docking site for Ets-1 phosphorylation by ERK2.

Comparisons to other docking sites

The ERK2 docking site in the Ets-1 and Ets-2 PNT domain (LXLXXXF) differs in sequence composition and position with respect to the phosphoacceptor compared to other MAPK docking sites. The critical role of the C-terminal phenylalanine in the Ets-1 and Ets-2 docking site distinguishes it from LXL docking motifs in the bZIP transcription factor c-Jun (Kallunki et al., 1996) and the ets proteins Elk-1 and SAP-1 (Yang et al., 1998a; Yang et al., 1998b; Galanis et al., 2001). Additionally, the Ets-1 lies docking site is approximately 80 residues C-terminal of a single phosphoacceptor, whereas the LXL docking motifs of c-Jun, Elk-1, and SAP-1 lie N-terminal to multiple phosphoacceptors (Kallunki et al., 1996; Yang et al., 1998a; Yang et al., 1998b; Galanis et al., 2001). The Ets-1 and Ets-2 docking site is also positioned differently than the FXF docking motif found in Elk-1 and SAP-1, as this site lies immediately C-terminal of a cluster of phosphoacceptors (Jacobs et al., 1999; Fantz et al., 2001; Galanis et al., 2001). Determination of the structural features of these docking sites would facilitate further functional and mechanistic comparisons.

The solution structure of the LXL motif containing ERK2 binding domain in the phosphatase MKP-3 was recently solved (Farooq et al., 2001). There are several differences in the structure and function of this domain compared to the PNT domain. First, the secondary structural elements and overall fold of this domain are distinct from the PNT domain. Second, the residues important for docking lie within different
structural elements than those in the PNT domain. Third, the MKP-3 docking site is proposed to interact with ERK2 outside the range of interfaces determined in our Ets-1 modeling experiment. Therefore, the MKP-3 LXL motif appears to function in a structurally and mechanistically distinct fashion than that of the LXL motif in the Ets-1 PNT domain.

**MAPK substrate specificity**

The docking function of the Ets-1 and Ets-2 PNT domain is expected to play a role in the specificity of MAPK phosphorylation. Besides ERKs, there are two other main classes of MAPKs, the p38 and JNK subfamilies. Preliminary *in vitro* experiments indicate that Ets-1 is a substrate for the MAPK p38, but the phosphoacceptor is not T38 (unpublished data). More importantly, mutation of the ERK2 docking site does not affect the efficiency of p38 phosphorylation. A JNK MAPK also phosphorylates Ets-2 at T72 (Smith et al., 2000); however, no kinetic analysis was performed. Further studies are required to understand the role of the docking site in determining the specificity of MAPK phosphorylation of Ets-1 and Ets-2.

**Specificity of function among the *ets* family of transcription factors**

Members of the *ets* family of transcription factors conserve the highly similar DNA binding ETS domain, yet regulate diverse sets of genes (Graves and Petersen, 1998). There are many molecular features that contribute to the unique functions of *ets* proteins. For instance, the PNT domain is conserved among approximately 40% of *ets* proteins, but can perform distinct functions in different proteins. The PNT domain has
been proposed to mediate homotypic oligomerization in TEL (Jousset et al., 1997), heterotypic oligomerization in TEL, Yan, Mae, and Pnt-P2 (Potter et al., 2000; Baker et al., 2001), interaction with the SUMO conjugating enzyme Ubc9 in TEL (Chakrabarti et al., 1999; Chakrabarti et al., 2000), and binding transcriptional corepressors in TEL (Fenrick et al., 1999; Wang and Hiebert, 2001). This study provides data for a novel role of the PNT domain as an ERK2 docking site. This docking function is apparent in the PNT domains of Ets-1 and Ets-2, and presumably *Drosophila* Pnt-P2. However, the docking sequences are not conserved in other PNT domains, and the PNT domain of GABPα does not efficiently dock with ERK2 in kinase assays. We speculate that an ERK docking site evolved within an ancestral ortholog of the genes encoding Ets-1, Ets-2, and Pnt-P2. This site enhances phosphorylation of the N-terminal phosphoacceptor site, thereby lending a unique function to two of 25 ets proteins in the human genome and 1 of 10 in the *Drosophila* genome.

The PNT domains of other proteins also are implicated in Ras/MAPK signaling. The PNT domain of the *Drosophila* protein Mae oligomerizes with the PNT domain of Yan and assists phosphorylation of Yan by the MAPK Rolled (Baker et al., 2001). It was speculated that the PNT domain of Mae interacts with Rolled; however, the Mae PNT domain lacks the docking site found in the PNT domain of Ets-1 and Ets-2 (Fig. 5.2). This suggests that among ets proteins the PNT domain may be able to regulate MAPK phosphorylation by multiple mechanisms.

In conclusion, our findings demonstrate how a structural domain, through changes in its surfaces residues, can establish varied functions among the ets gene family members. This diversification has facilitated the utility of different family
members in regulating the complicated arrays of genomic information in higher eukaryotes. Thus, this study sheds new light on the role of families of DNA binding proteins in the metazoa.

**Materials and methods**

**Plasmid construction**

Bacterial expression plasmids encoding histidine-tagged murine Ets-1(1-138) (described previously, (Slupsky et al., 1998)), Ets-1(1-52), Ets-2(1-172), and Ets-1(1-50)/GABPα(167-254) were generated by PCR-amplification of the appropriate cDNA and subcloning into the *Nde* I and *Hind* III sites of pET28a(+) (Novagen). The mammalian expression plasmid pEVRFO-Ets-1 was described previously (Nelsen et al., 1993). The mammalian expression plasmid encoding Ets-2 was generated by subcloning the murine Ets-2 open reading frame (ORF) into the *BamH* I site of the pEVRFO plasmid (Matthias et al., 1989). Site-directed mutagenesis was conducted using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). Mammalian expression plasmids encoding FLAG-epitope tagged ets proteins were generated by PCR-amplification of the ORFs of pEVRFO plasmids encoding wild-type or mutant Ets-1 and Ets-2 followed by subcloning into the *Pst* I and *Apa* I site of pCMV-Tag2a (Stratagene). The luciferase reporter was constructed by inserting the Ras responsive element spanning −527 to −544 of the matrix metalloproteinase (MMP)-9 gene (Watabe et al., 1998) into the *Mlu* I and *Nhe* I site of the pGL3-basic plasmid (Promega) containing 72 bp of the rat prolactin minimal promoter (Ingraham et al., 1988).
Expression and purification of ets proteins

Full-length murine Ets-1 was expressed and purified as previously described (Cowley and Graves, 2000) with the addition of a final purification step. Following S-Sepharose chromatography, Ets-1 was dialyzed in Buffer A [25 mM Tris-HCl, pH 7.9, 10% (vol/vol) glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 150 mM KCl, and 1 mM dithiothreitol (DTT)], and purified on a Sephacryl-200 size exclusion column (Amersham-Pharmacia) in the same buffer.

To express histidine-tagged ets proteins, the appropriate plasmids were transformed into Escherichia coli BL21(DE3). Transformants were used to inoculate overnight cultures in 50 ml Luria Broth (LB), 25 μg/ml kanamycin, and 0.08% glucose. Overnight cultures were subcultured 1:200 in LB containing 25 μg/ml kanamycin and grown at 30°C or 37°C. Protein expression was induced by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at an A₆₀₀ of 0.5-0.8. Cells were grown for an additional three to five hours, harvested by centrifugation, frozen in liquid nitrogen, and stored at -80°C until purification.

To purify histidine-tagged ets proteins, frozen cell pellets were thawed at 37°C for approximately 5 min, resuspended in buffer containing 50 mM sodium phosphate, pH 8.0, 100 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 1 mg/ml lysozyme, lysed by sonication (Heat Systems, Inc.) and the cellular debris was pelleted by ultracentrifugation (Beckman) in a 50.2 Ti rotor at 40,000 rpm, 30 min, 4°C. The supernatant containing soluble ets proteins was filtered through a Nalgene 0.2 μ bottle top filter, and imidazole was added to a final concentration of 5 mM.
Chromatography was performed on an Äkta FPLC (Amersham-Pharmacia). Soluble extract was passed over a 5 ml HiTrap chelating column (Amersham-Pharmacia) charged with nickel sulfate and equilibrated with 50 mM sodium phosphate, pH 8.0, 100 mM KCl, and 5 mM imidazole. Histidine-tagged ets proteins were eluted with a gradient of imidazole. Fractions containing ets proteins as verified by SDS-PAGE were pooled, dispensed into 12,000-14,000 MWCO dialysis tubing (Spectra), and dialyzed against 2 liters of buffer containing 25 mM Tris-HCl, pH 7.9, 10% glycerol, 0.1 mM EDTA, 150 mM KCl, 1 mM DTT, and 0.2 mM PMSF for 2 hours followed by a buffer change and overnight dialysis. Following overnight dialysis, ets proteins were concentrated using Centriprep YM-10 concentrators, filtered through a 0.2 μ syringe filter, and purified on a 16/60 Superdex-75 size exclusion column (Amersham-Pharmacia) equilibrated with 25 mM Tris-HCl, pH 7.9, 10% glycerol, 0.1 mM EDTA, 150 mM KCl, and 1 mM DTT. Fractions containing ets proteins as verified by SDS-PAGE were pooled, concentrated with CentriprepYM-10 concentrators, and filtered through a 0.2 μ syringe filter.

The concentration of the protein was determined by absorbance at 280 nm using extinction coefficients as determined by the method of Gill and von Hippel (Ets-1(1-138) and mutants thereof: ε = 23,240 M⁻¹cm⁻¹, except C112A (ε = 23,120 M⁻¹cm⁻¹); Ets-2(1-172) and mutants thereof: ε = 28,930 M⁻¹cm⁻¹) (Gill and von Hippel, 1989); guanidine-HCl was not used for concentration determination, as it had minimal affects on absorbance readings. The concentration of Eis-1(1-52) was determined by the method of Bradford (Bio-Rad). Mass spectrometry and amino-terminal sequencing of several mutants indicated the first methionine of the histidine tag is cleaved. Purified
proteins were dispensed into single-use aliquots, frozen in liquid nitrogen, and stored at -80°C.

Expression and purification of activated ERK2

To express activated ERK2, BL21 (DE3) cells were freshly transformed with a plasmid encoding histidine-tagged rat ERK2 and a constitutively activate mutant of human MEK1 (generous gift of Melanie Cobb, (Khokhlatchev et al., 1997)), and a single colony was used to inoculate 100 ml Terrific Broth (TB) containing 100 µg/ml ampicillin. Following growth overnight at 37°C, the culture was diluted 1:40 in TB, 100 µg/ml ampicillin, and grown at 30°C. Protein expression was induced by adding IPTG at a final concentration of 0.3 mM at an A600 of approximately 0.3, and cells were grown for 21 hours. Cells were harvested by centrifugation at 5000g for 10 min, resuspended in 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, pelleted, frozen in liquid nitrogen and stored at −80°C until purification.

To purify activated ERK2, cell pellets were thawed and resuspended in 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, 2 µg/ml aprotinin, 2 µg/ml leupeptin, 0.7 µg/µl pepstatin, 0.2 mM PMSF, 0.3% NP-40, and 1 mg/ml lysozyme. The resuspended cells were lysed by sonification, and the cellular debris was pelleted by centrifugation. Soluble extract was loaded on a 1 ml HiTrap chelating column (Amersham-Pharmacia) charged with nickel sulfate and equilibrated with 50 mM sodium phosphate, pH 8.0, 300 mM sodium chloride, and 20 mM imidazole. ERK2 was eluted from the column using a gradient of imidazole. Fractions containing ERK2 as verified by SDS-PAGE were pooled, dispensed into 12,000-14,000 MWCO dialysis bags, and dialyzed overnight against 25 mM Tris-HCl, pH 7.5, 10% glycerol, 1 mM
EDTA, 0.2 mM EGTA, and 50 mM KCl. Following dialysis, particulate was removed by centrifugation, ERK2 was bound to a HR5/5 Mono Q column, and was eluted with a gradient of potassium chloride. The purest fractions, as assessed by SDS-PAGE, were dialyzed against 2 liters of 25 mM Tris-HCl, pH 7.5, 20% glycerol, 0.1 mM EDTA, 50 mM KCl, and 1 mM DTT. ERK2 was diluted to 100 nM as determined by Bradford assay (Bio-Rad), aliquoted, frozen in liquid nitrogen, and stored in −80°C. Activated ERK2 retained its activity for over a year.

**Kinase assays**

Kinase reactions were performed in buffer containing 25 mM Tris-HCl, pH 7.9, 87.5 mM KCl, 5% glycerol, 10 mM magnesium acetate, 0.5 mg/ml bovine serum albumin, 10 mM DTT, 2 mM ATP (Amersham-Pharmacia), 25 mCi/mmol [γ-³²P] ATP (ICN), and 0.1 nM ERK2 in a total volume of 20 μl. ATP was in excess (>10x the estimated $K_m(\text{ATP})$). Reactions were performed at 30°C and were initiated by the addition of ATP. After 15 min, reactions were stopped by the addition of 3X SDS sample buffer and electrophoresed on a SDS-polyacrylamide gel. Gels were stained with Coomassie Blue, dried on Whatman paper, and exposed to a phosphor screen. Radioactivity was detected by phosphorimaging (Storm 860, Molecular Dynamics) and quantified relative to an ATP standard curve using ImageQuant software (v.5.0 Molecular Dynamics). Less than 15% of ets protein was phosphorylated, and phosphate incorporation was linear during the experiment. Initial reaction velocities ($v$) were determined by dividing product concentration by the time of the reaction. $K_m$, the Michaelis constant, and $k_{cat}$, the turnover number, were determined by fitting $v/[E]_0$ ($[E]_0$, total ERK2 concentration) from two to five independent experiments performed.
in duplicate to the Michaelis-Menten equation \( \frac{v}{[E]_0} = \frac{k_{cat}[S]}{(K_m + [S])} \), where \([S]\) is the protein concentration, by nonlinear least squares analysis (Kaleidagraph v.3.0).

**Transient transfection and luciferase assay**

NIH3T3 cells (generous gift of Peggy Farnham) were grown in DMEM (Dulbecco modified Eagle medium) with 10% bovine calf serum (BCS). Cells (1.5 x \(10^5\)) were plated in 35 mm tissue culture dishes and were transfected with calcium phosphate precipitates of plasmids encoding wild-type or mutant versions of Ets-1 and Ets-2, a plasmid encoding a constitutively active version of MEK1 (\(\Delta N3;S218E;S222D\)) (Mansour et al., 1994), an internal control plasmid pRL-null (Promega), and the MMP-9 luciferase reporter. Twenty to 24 hr after transfection the media was changed to DMEM containing 0.5% BCS and the cells were incubated for an additional 20-24 hr. Firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) on a Dynex Technologies, Inc., MLX Microtiter Plate Luminometer. Firefly luciferase activity was normalized to Renilla luciferase activity. Each transfection was performed in duplicate and relative luciferase activity (RLA) was reported. The experiment was performed twice with \(ets\) protein lacking FLAG-tags and twice with FLAG-tagged \(ets\) proteins; presence of the FLAG tag did not significantly affect results.

To detect expression of \(ets\) proteins, 5 x \(10^5\) NIH3T3 cells plated in 60 mm tissue culture dishes were transfected with calcium phosphate precipitates of 20 \(\mu\)g of expression plasmid encoding wild-type or mutant FLAG-epitope tagged \(ets\) protein. Twenty hr following transfection, cells were metabolically labeled for 15 hr in 1.5 ml of DMEM (minus methionine) containing 0.5% BCS, 2 mM glutamine, and 150 \(\mu\)Ci of...
[³⁵S] methionine (Amersham Pharmacia). Cells were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, and the complete, mini protease inhibitor cocktail (Roche). Lysis was assisted by scraping cells into an eppendorf tube, incubating on ice, and rotating at 4°C for 30 min. Cellular debris was pelleted by centrifugation, and the supernatant was added to approximately 10 ul of ANTI-FLAG M2 Affinity Gel (Sigma) pre-washed in Tris buffered saline (TBS).

Immunoprecipitation was allowed to occur for 30.5 hours while rotating at 4°C. Beads were washed 4 times with 250 µl TBS. Proteins were eluted by the addition of 15 µl of 3x SDS-sample buffer and boiling for 10 min and were electrophoresed on a 10% SDS-polyacrylamide gel. Gels were dried on Whatman paper and exposed to a phosphor screen. Radioactive proteins on dried gels were detected by phosphorimaging and quantified relative to background bands using ImageQuant software.

**Partial trypsin proteolysis sensitivity assays**

Ets-1(1-138) or Ets-1(i-138;L114R;L116R;F120A) (110 µg each) were incubated with 5.5 µg trypsin in a total reaction volume of 55 µl at 22.5°C. Aliquots of 5 µl (10 µg total ets protein) were taken at various time intervals, added to 3X SDS sample buffer, and heated at >95°C for 5 min to stop the proteolysis reaction.

Proteolyzed fragments were electrophoresed on an 18% SDS-polyacrylamide gel and were detected by Coomassie Blue staining. To identify sites of proteolysis, a similar assay was performed and the pool of fragments was transferred to PVDF for N-terminal sequencing.
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CHAPTER 6

SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS
Evolutionary perspective of the ets gene family

Members of the ets family of DNA binding proteins are found throughout all the metazoa and appear to have originated in an early multicelled ancestor. It is conceivable that as multicelled organisms became more complex, multiple ets proteins evolved to control increased amounts of genetic information. Through evolution and speciation, though, individual ets proteins evolved distinct functions. Underlying these distinct functions are numerous molecular changes. For instance, changes in regulatory elements in the promoters and enhancers of ets genes resulted in different temporal and spatial expression patterns. Certain residues of the ETS domain diverged, while residues necessary for defining the winged-helix-tum-helix fold were conserved. Ets proteins accrued, and possibly lost, other domains, such as the PNT domain. Sites of post-translational modifications arose to regulate the activity of these proteins. These changes establish the family as it exists today.

Summary of PNT domain studies

My thesis work investigated the function of the PNT domain and sites of post-translational modification to gain insight into the determinants of the unique molecular functions among the ets family of transcription factors. The work described in this dissertation examined the structure and function of the PNT domains of the ets proteins TEL, Ets-1, and Ets-2 as well as the regulation of MAPK phosphorylation of Ets-1 and Ets-2. Several lines of experimentation revealed four major findings regarding the function of these entities. 1) NMR and biochemical analysis were used to determine the structure of the Ets-1 PNT domain and N-terminal MAPK site (Chapter 2; Slupsky et al., 1998). This work was performed primarily by Lawrence McIntosh’s laboratory; I
contributed a partial proteolysis experiment. 2) Biochemical analysis demonstrated that the TEL PNT domain can multimerize, a phenomenon subsequently explained as polymerization (Chapter 3; Kim et al., 2001). 3) An affinity chromatography experiment demonstrated that the Ets-1 PNT domain interacts with multiple protein partners from a calf thymus nuclear extract. Two proteins were identified by a candidate approach to be UBC9 and ERK1/2 (Chapter 4). 4) Enzymatic and transient transfection studies revealed that the Ets-1 and Ets-2 PNT domains contain an ERK2 docking site that enhances phosphorylation of an N-terminal MAPK phosphoacceptor. The docking site is required for Ras pathway mediated enhancement of the transactivation activity of Ets-1 and Ets-2. The docking site does not appear to be conserved in the PNT domains of all family members (Chapter 5). These results have important implications for the mechanism of function of ets proteins and MAPKs and the evolutionary relationships among ets proteins.

Discussion

Sequence and structural similarities define the PNT domain

Although sequence similarity originally defined the PNT domain, X-ray crystallographic and NMR studies demonstrate that structural features also define this domain. The Ets-1 and TEL PNT domains are composed of a core of four α-helices that assume similar folds (Slupsky et al., 1998; Kim et al., 2001). Sequence similarity in the core helices predicts that these elements and their fold are conserved in all PNT domains. Ets-1 contains an additional helix (H1) N-terminal to the core helices that may or may not exist in other PNT domains.
Structural similarity: The SAM/PNT domain is a (HhH)₂ motif

The sequences defining helices H2-H5 of the PNT domain have weak similarity to sequences defining the sterile-α-motif (SAM) domain. A stronger relationship between PNT domains and SAM domains was established through structural studies. The SAM domains of the receptors EphA4 (Stapleton et al., 1999) and EphB2 (Smalía et al., 1999; Thanos et al., 1999a; Thanos et al., 1999b), and the transcription factor p73 (Chi et al., 1999; Wang et al., 2001) are composed of five helices that fold into a structure that closely resembles the fold of helices H2-H5 of PNT domains. Due to these sequence and structural similarities, these domains are often collectively referred to as the SAM/PNT domain.

The SAM/PNT domain shares structural elements that define a larger class of motifs termed the helix-hairpin-helix (HhH) domain (Fig 6.1; Shao and Grishin, 2000). The SAM/PNT domain is an example of an (HhH)₂ domain, which has a five helix architecture composed of two repeated HhH motifs connected by a helix. The HhH domain is found in diverse proteins, including RuvA, Rad51, DNA-polymerases, DNA helicases, 5' to 3' exonucleases, and RadA. Unlike the proposed functions for the SAM/PNT domain, the (HhH)₂ motifs of other proteins often mediate non-sequence specific DNA binding. Despite this difference in function, the structural fold of SAM domains, PNT domains, and other (HhH)₂ motifs appears to have been derived from a common evolutionary ancestor.
Figure 6 HhH motif. Comparison of the secondary structural elements of the Ets-1 PNT domain and EphB2 SAM domain. Rectangles represent helices. HhH motifs are underlined; H3 is a connector helix. The first HhH motif in Ets-1 is not readily apparent; however, comparison of three dimensional structures establishes a clearer relationship to the (HhH)$_2$ motif.
The SAM/PNT domain as a protein interaction motif

Although homotypic dimerization, polymerization, and heterotypic associations are functions for many SAM/PNT domains, such associations are not a defining feature of all domains. The SAM/PNT domains of Ets-1 (Slupsky et al., 1998), Ets-2, Fli-1 (Kim et al., 2001), and GABPα (L. McIntosh, personal communication), for example, are monomeric and have not been reported to interact with other SAM/PNT domains. It is possible, however, that other SAM/PNT domain partners for these SAM/PNT domains exist but have not yet been identified.

Another intriguing possibility, although not yet observed, is that SAM domains could interact with PNT domains from ets proteins. For instance, polycomb proteins, which lack DNA binding domains, could interact with PNT domains of ets proteins. The DNA binding domain of ets proteins could be used to target polycomb proteins to specific genes.

A role for SAM/PNT domains in signal transduction

The work described in this dissertation revealed a novel function for the PNT domain as an ERK2 interaction motif. Not all PNT domains appear to contain the ERK2 docking site found in Ets-1 and Ets-2, as suggested by sequence lineups and functional studies. Thus, it was argued in Chapter 5 that the docking site might be specifically found in the Ets-1, Ets-2, and Pnt-P2 PNT domains due to the presence of the N-terminal MAPK phosphoacceptor.

Despite apparently limited conservation of the docking site, it is striking that SAM/PNT domains are directly or indirectly connected to signal transduction pathways in other ets and non-ets proteins. For instance, the PNT domains of Drosophila Mae,
Pnt-P2, and Yan are proposed to form a direct interaction with the *Drosophila* MAPK Rolled (Baker et al., 2001). Also, although SAM domains have not been reported to interact with MAPKs, proteins with SAM domains are often involved in signaling pathways, such as tyrosine and serine/threonine protein kinases, cytoplasmic scaffolding and adaptor proteins and GTPases (Schultz et al., 1997). It is possible that an ancestral SAM/PNT domain may have evolved to perform a specific function in signaling pathways. It is also tempting to speculate that PNT domains may have been introduced into *ets* proteins as a means of establishing a connection between these proteins and signaling pathways.

**Specificity of MAPK phosphorylation of Ets-1, Ets-2, and Pnt-P2**

The MAPK docking site may determine which kinases phosphorylate T38 and T72, in Ets-1 and Ets-2, respectively. In other transcription factors, docking sites differentially target MAPKs to substrates (Sharrocks et al., 2000). This precedent may hold for the MAPK docking site in the Ets-1 and Ets-2 PNT domains. Interestingly, it was reported that the cyclin-dependent kinase CDK10 bound a PNT domain containing fragment of Ets-2 (Kasten and Giordano, 2001). However, CDK10 was not reported to phosphorylate Ets-2, so the role of the interaction is not known.

**Mechanism of phosphorylation-mediated enhancement of transactivation**

Phosphorylation of the N-terminal MAPK site in Ets-1 and Ets-2 could enhance transactivation by multiple mechanisms. Phosphorylation could affect protein partnerships with other transcription factors, chromatin remodeling machinery, or the basal transcriptional machinery. For example, unphosphorylated Ets-1 and Ets-2 may
interact with corepressors. Phosphorylation could disrupt these interactions, thereby releasing a repression complex. In contrast, phosphorylation may recruit a coactivator protein or complex to Ets-1 or Ets-2. A combination of the two phenomena is possible. However, phosphorylation does not appear to affect DNA binding activity or nuclear localization (B. Colson, C. Foulds, unpublished data).

**Mechanism of action of the ERK2 docking sites**

The mechanism by which docking sites enhance the efficiency of phosphorylation of a substrate is not known. It is postulated that dockings sites interact with kinases outside the catalytic site, and several sites have been identified (Tanoue et al., 2000; Tanoue et al., 2001; Xu et al., 2001). These interactions are thought to increase the local concentration of the phosphoacceptor near the catalytic site. However, the interaction model has not been fully demonstrated, due primarily to a lack of structural information for MAPK/substrate interactions.

**Future directions**

The experiments described within this dissertation serve as the framework for multiple future investigations into ets protein function. The studies proposed below address the molecular features of Ets-1 that allow it to function as a transcriptional activator and as an integrator of inputs from extracellular signal transduction pathways.

**Identify other protein partners of the Ets-1 PNT domain**

It is likely that other proteins besides ERK2 and UBC9 interact with the Ets-1 PNT domain. Consistent with this hypothesis, it was estimated that approximately 17 proteins from the calf thymus nuclear extract bound the Ets-1(29-138) affinity column.
In addition to identifying these partners, the affinity chromatography experiment could be performed with a different source of potential partners, such as a HeLa cell extract. Alternatively, affinity chromatography could be performed with a PNT domain from a *C. elegans ets* protein, using extract from *C. elegans* as a source of potential partners. Because the *C. elegans* genome is sequenced, protein partners could be more easily identified by available techniques, such as mass spectrometry.

**Determine mechanism of MAPK mediated enhancement of transactivation**

There are several protein interaction assays that could be used to identify partners of phosphorylated Ets-1. First, affinity chromatography could be performed with a phosphorylated fragment of Ets-1. Second, a cDNA expression library could be probed with phosphorylated Ets-1. Third, a yeast two-hybrid interaction assay could be attempted with co-expressed and constitutively active MEK1 and ERK2. Preliminarily identified interactions could be confirmed by a variety of assays.

**Structural studies of the Ets-1/ERK2 interaction**

High-resolution structural information and other biochemical studies would provide support for the Ets-1/ERK2 interaction. X-ray crystallography could be undertaken to determine the co-crystal structure of the MAPK with Ets-1. NMR titration experiments could be performed with $^{15}$N labeled Ets-1 fragments to further define the residues on Ets-1 required for docking with ERK2. The docking interaction surface on ERK2 could be mapped through competition assays with peptides of surface helices of ERK2. Alternatively, candidate surface residues of ERK2 could be chosen for mutagenesis to determine the effect of mutations on docking.
MAPK docking of Pnt-P2 in *Drosophila*

Genetic studies of the role of Pnt-P2 in *Drosophila* eye development could be used to provide *in vivo* evidence of the function of MAPK docking. Pnt-P2 is highly related to Ets-1 and Ets-2, and evidence suggests that Ets-1, Ets-2, and Pnt-P2 are functionally redundant. For instance, MAPK phosphorylated Pnt-P2 functions similarly to phosphorylated Ets-1 and Ets-2 in transfection reporter assays, and Ets-1 expressed in *Drosophila* during development can rescue mutant phenotypes caused by null alleles of *pnt* (Albagli et al., 1996). Docking site mutants of Pnt-P2 or Ets-1 could be introduced into flies to determine if docking is required for R7 photoreceptor development.

**Phosphorylation specificity of the N-terminal MAPK site**

The specificity of MAPK phosphorylation of Ets-1 and Ets-2 could be tested by a combination of *in vitro* and *in vivo* experiments. A battery of purified MAPKs and CDKs could be used to attempt to phosphorylate wild-type and ERK2 docking site mutant forms of Ets-1. Enzymatic assays could be used to quantitate the efficiency of phosphorylation. These studies could be collaborated by transient transfection reporter assays. These assays would make use of constructs expressing different MAPKs and CDKs to determine the effects of kinases on the transactivational activities of Ets-1.

**Final summary**

In summary, the studies described within this dissertation identified a novel function for the PNT domain of Ets-1 and Ets-2 as an ERK2 docking site. This work is the first description of the structural context of a MAPK docking site within a transcription factor, and also details the position of the docking site with respect to the
phosphoacceptor in three-dimensional space. This thesis has provided insight into how MAPK phosphorylation of Ets-1 is regulated and how specific PNT domains perform unique functions. These results serve as a framework for future studies investigating the specificity of function among ets family members as well as the mechanism of MAPK docking interactions.

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