Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival

Lianping Xing,[1,5] Ana M. Venegas,[2,5] Amy Chen,[2] Lisa Garrett-Beal,[2] Brendan F. Boyce,[1] Harold E. Varmus,[3,4] and Pamela L. Schwartzberg[2,3,6]

[1]Department of Pathology, University of Rochester, Rochester, New York 14627, USA; [2]National Human Genome Research Institute and [3]National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20892, USA

Mutant $src^{-/-}$ mice have osteopetrosis resulting from defective osteoclasts, the cells that resorb bone. However, signaling pathways involving Src family members in osteoclasts remain unclear. We demonstrate that expression of a truncated Src molecule, Src251, lacking the kinase domain, induces osteopetrosis in wild-type and $src^{+/-}$ mice and worsens osteopetrosis in $src^{-/-}$ mice by a novel mechanism, increased osteoclast apoptosis. Induction of apoptosis by Src251 requires a functional SH2, but not an SH3, domain and is associated with reduced AKT kinase activity. Expression of Src251 dramatically reduces osteoclast survival in response to RANKL/TRANCE/OPGL, providing evidence that Src family kinases are required in vivo for survival signaling pathways downstream from TNF family receptors.

The c-Src tyrosine kinase has been implicated in multiple signaling pathways that regulate cellular growth, migration, and survival (Brown and Cooper 1996; Thomas and Brugge 1997). First defined as the normal cellular counterpart of the transforming gene of Rous Sarcoma Virus, c-*src* is overexpressed in a variety of human tumors, and an activating mutation has recently been found in a subpopulation of colon carcinomas (Bolen et al. 1987; Cartwright et al. 1989; Irby et al. 1999). Src kinase activity increases in response to a number of signaling pathways, most notably those downstream from tyrosine kinase growth factor receptors, G-protein coupled receptors, and integrin cell surface adhesion molecules (Brown and Cooper 1996; Thomas and Brugge 1997). Recent data suggest that Src can be activated downstream from RANK (receptor activator of NF-κB/osteoprotogerin-ligand-receptor/TRANCE-receptor) (Wong et al. 1999a), a member of the tumor necrosis factor (TNF) receptor superfamily that is involved in osteoclast formation and survival, as well as in osteoclast and dendritic cell function (Fuller et al. 1998; Dougall et al. 1999; Wong et al. 1999b; Li et al. 2000). The association of Src with these multiple signaling pathways

makes it a potential therapeutic target for several diseases, including osteoporosis and cancer.

Src is a member of a family of nonreceptor tyrosine kinases defined by a common structure (see Fig. 1) that includes a myristoylated unique amino-terminal domain followed by the Src homology (SH) protein interaction domains: the SH3 domain interacts with proline-rich regions, and the SH2 domain interacts with phosphorylated tyrosine residues (Pawson and Gish 1992; Brown and Cooper 1996). The tyrosine kinase catalytic domain comprises the second half of the molecule and is followed by a short carboxy-terminal tail containing a negative regulatory tyrosine (Y527). This tyrosine is normally phosphorylated by the CSK kinase, causing an intramolecular interaction with Src's own SH2 domain that leads to a closed conformation and inactivation of the kinase (see Fig. 7C below; Brown and Cooper 1996). Mutational and crystallographic data suggest that a second intramolecular interaction, between the SH3 domain and the region that links the SH2 domain to the kinase, is also important for kinase regulation (Jove and Hanafusa 1987; Parsons and Weber 1989; Yamaguchi and Hendrickson 1996; Sicheri et al. 1997; Xu et al. 1997). Together, these intramolecular associations closely control Src kinase activity, but they also regulate the availability of the SH2 and SH3 domains for interactions with other molecules and influence the subcellular localization of Src, so that activated Src becomes detergent-insoluble.

The widespread expression of c-*src*, its exquisite regulation, and the many pathways that activate this kinase,

[4]Present address: Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA.
[5]These authors contributed equally to this work.
[6]Corresponding author.
E-MAIL pams@nhgri.nih.gov; FAX (301) 402-2170.
Article and publication are at www.genesdev.org/cgi/doi/10.1101/gad.840301.
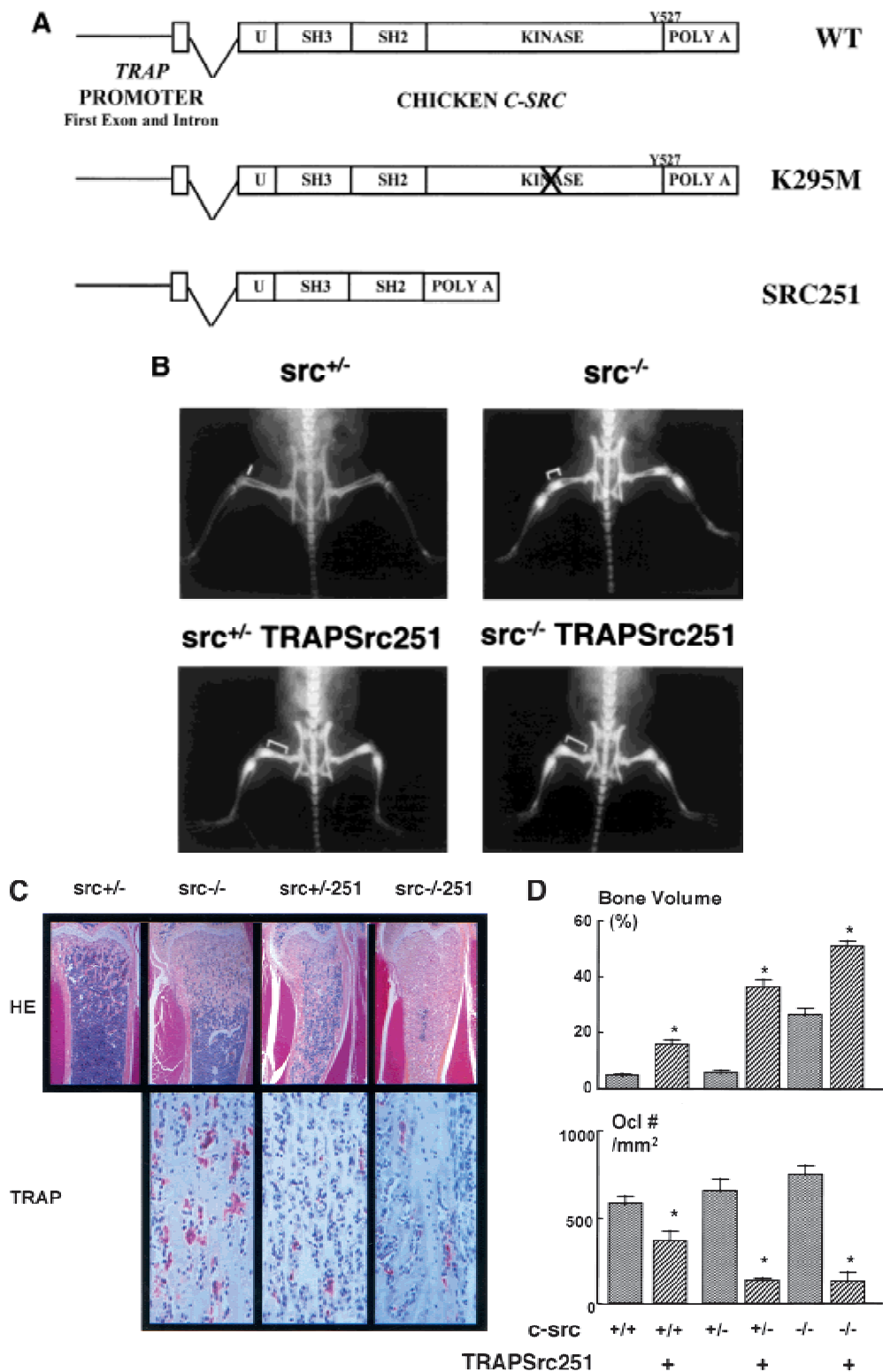
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Figure 1. Src251 induces osteopetrosis. (*A*) Structure of *TRAPSrc* transgenic constructs. The *TRAP* promoter, first exon, and intron were fused to the ATG of wild-type (WT) and mutant versions of the chicken c-*src* gene. (U) Unique region; (SH3) Src homology 3 region; (SH2) Src homology 2 region; (Kinase) kinase domain. Crosses overlay domains containing inactivating mutations. (*B*) Radiographic analyses of Src251 mice. X-rays, frontal view. Brackets indicate regions that demonstrate increased bone density. (*C*) Histological analyses of tibia. Sections were stained both with Hematoxylin and Eosin (H&E) and for TRAP activity. (Upper panel) low power (4× magnification). (Lower panel) intermediate power (20×), showing TRAP-positive osteoclasts. (*D*) Histomorphometric analyses of mice. Genotype is indicated on the X axis. Percentage bone volume (amount of bone matrix as a percentage of the cancellous bone) is indicated on the Y axis. Osteoclasts were counted as TRAP-positive cells.

suggest that Src and Src-family kinases are critical for normal cellular function.

Nonetheless, targeted disruption of the c-*src* gene in mice leads to only one predominant phenotype, osteopetrosis, or a failure to break down bone, resulting from an intrinsic functional defect in osteoclasts, a cell type that expresses high levels of Src (Soriano et al. 1991; Horne et al. 1992; Lowe et al. 1993; Lowell et al. 1996). The *src*^−/− mice develop osteoclasts, but the cells are morphologically abnormal, having aberrant cytoskeletal organization and lacking ruffled borders, the polarized part of the cell responsible for bone resorption (Boyce et al. 1992; Insogna et al. 1997; Schwartzberg et al. 1997). Accordingly, *src*^−/− osteoclasts fail to resorb bone effectively and show defective phosphorylation of cellular proteins in response to extracellular stimuli, such as M-CSF and engagement of integrin receptors (Tanaka et al. 1996; Insogna et al. 1997; Duong et al. 1998; Nakamura et al. 1998). Recent in vitro data suggest that Src may be activated by a RANK-mediated cell-survival pathway in osteoclasts (Wong et al. 1999a). However, to date, there is no evidence of decreased osteoclast survival in *src*^−/− mice in vivo. Indeed, *src*^−/− mice have normal to elevated numbers of osteoclasts (Boyce et al. 1992).

Although the phenotype associated with Src deficiency may appear relatively limited, osteoclasts present a unique, complex cell type in which to examine Src function in a physiological setting. To investigate the multiple possible functions of Src in the osteoclast, we have undertaken a genetic dissection of Src function by reexpressing wild-type (wt) and mutated versions of c-*src* in osteoclasts of Src-deficient mice under the control of the promoter for tartrate-resistant acid phosphatase (TRAP), a gene that is highly expressed in osteoclasts. Using this system, we have shown previously that expression of a wild-type chicken c-*src* cDNA can fully complement *src*^−/− osteopetrosis (Schwartzberg et al. 1997). Surprisingly, expression of a kinase-defective point mutant of Src (*SrcK295M*) also rescued many aspects of the osteopetrosis resulting from Src deficiency. Combined with experiments in *src*^−/− fibroblasts indicating that Src kinase activity may not be strictly required for signaling from integrin receptors (Kaplan et al. 1995; Schlaepfer et al. 1998; P.L. Schwartzberg, unpubl.), these results pose the possibility that in certain pathways Src may function, in part, as an adaptor molecule.

To further examine the requirements for Src function in the osteoclast, we constructed a transgene, *Src251*, which drives expression of the amino-terminal 251 amino acids of Src, lacking the entire kinase domain and the regulatory tyrosine 527. Surprisingly, the *Src251* transgene induced osteopetrosis in wild-type and *src*^+/− mice and worsened osteopetrosis in *src*^−/− mice. Osteopetrosis in *Src251* mice was associated with a novel phenotype not observed in *src*^−/− mice: increased osteoclast apoptosis. We present genetic and biochemical evidence indicating that this apoptosis may be caused by misregulation of the Src251 protein interaction domains. Expression of *Src251* was associated with decreased AKT kinase activity and decreased osteoclast survival in re-

sponse to RANKL, providing in vivo evidence for regulation by Src family kinases of cell-survival pathways involving TNF family receptors and AKT. These results further indicate that proper regulation of Src protein interaction domains is a critical element of Src function in vivo and may be a particularly effective target for Src-based therapeutics.

Results

Src251 induces osteopetrosis

We have demonstrated previously that expression of either wild-type or a kinase-defective point mutant of Src in osteoclasts of transgenic mice improved bone histology in many *src*^−/− animals (Schwartzberg et al. 1997). To further examine the requirement for the kinase domain in osteoclast function, we constructed a transgene in which the first 251 amino acids of chicken c-Src, Src251, including the unique region and SH2 and SH3 domains, but lacking the kinase domain and regulatory tyrosine (Y527), was expressed under the control of the *TRAP* promoter (Fig. 1A).

In contrast to the full-length kinase-defective Src mutant (*SrcK295M*), which has no effect in a wild-type background, expression of the *Src251* transgene caused a failure of tooth eruption. This is one of the most obvious manifestations of osteopetrosis, resulting from a failure of osteoclasts to erode a channel for erupting teeth (Soriano et al. 1991). Progeny from several founders revealed varying degrees of penetrance of this dental phenotype, with multiple transgenic lines showing a lack of tooth eruption in both wild-type and heterozygous backgrounds (Table 1). An additional line in which tooth eruption was normal did not express the transgene, confirming that the phenotype correlated with expression of *Src251*. Interbreeding with c-*src* heterozygous mice to generate *src*^−/−*TRAPsrc*^251 mice further demonstrated that this transgene was unable to complement osteopetrosis in *src*^−/− mice.

Radiological examination confirmed increased bone density typical of osteopetrosis in *src*^+/+ and *src*^+/− mice expressing the *Src251* transgene (Fig. 1B). Surprisingly,

Table 1. *Induction of osteopetrosis by* TRAPSrc^251 *transgene*

Transgenic line	c-src	% with tooth eruption
—	+/−	100%
—	+/−	100%
—	−/−	0%
Src^251V	+/+	18%
Src^251V	+/−	0%
Src^251V	−/−	0%
Src^251Y	+/+	70%
Src^251Y	+/−	43%
Src^251Y	−/−	0%
Src^251Z	+/+	96%
Src^251Z	+/−	2%
Src^251Z	−/−	0%

however, osteopetrosis in $src^{-/-}$ mice was also exacerbated significantly by expression of *Src251*. Histologically, variable degrees of osteopetrosis were observed both in long bones and vertebrae of *Src251* mice, with significant increases in bone volume in all c-*src* genotypes (Fig. 1C,D; data not shown). The increased osteopetrosis in Src-deficient animals indicated that expression of the *Src251* transgene caused a more profound phenotype than Src deficiency alone. Thus, Src251 may interfere with more pathways than does the lack of Src, perhaps by interfering with the actions of other Src family kinases expressed in osteoclasts.

Src251 induces apoptosis in osteoclasts

Several mouse models of osteopetrosis, including mice with mutations in the genes encoding c-Fos, PU.1, CSF-1, RANKL (osteoprotogerin ligand/TRANCE), RANK, and the NF-κB subunits p50 and p52, result from a lack of osteoclast development (Wiktor-Jedrzejczak et al. 1990; Yoshida et al. 1990; Wang et al. 1992; Franzoso et al. 1997; Tondravi et al. 1997; Dougall et al. 1999; Kong et al. 1999b; Li et al. 2000). In contrast, $src^{-/-}$ mice develop normal to increased numbers of osteoclasts, but their function is defective (Boyce et al. 1992).

Src251 transgenic mice, however, had reduced numbers of osteoclasts (Fig. 1C,D), and many of these cells exhibited the classic morphological features of apoptosis, including pyknotic, fragmented nuclei and condensed cytoplasm. These features were readily visible in TRAP-stained sections (Fig. 2A) and were confirmed by TUNEL staining (data not shown).

Significantly, the percentage of apoptotic osteoclasts correlated with the degree of osteopetrosis and was inversely correlated with the levels of endogenous Src present in the wild-type and heterozygous mice (Fig. 2B). Culture of bone marrow or splenocytes from *Src251* mice in vitro resulted in generation of osteoclasts, unlike the results from osteopetrotic strains that lack osteoclast development. However, cultures from *Src251* mice yielded significantly fewer osteoclasts than those from either wild-type or $src^{-/-}$ mice, and significantly more of the osteoclasts were apoptotic (data not shown; see Fig. 6 below). Thus, Src251 may cause osteopetrosis by a novel mechanism, that of inducing osteoclast apoptosis. These data suggest that Src and/or other Src family members may be involved in cell signaling pathways that are required for cell survival in the osteoclast.

Abnormal localization of Src251 in osteoclasts

Src251 lacks kinase activity as a result of the absence of the kinase domain. However, Src251 appears to elicit a quite different phenotype from the full-length kinase-defective point-mutant, *SrcK295M*, which can partially rescue certain of the defects associated with Src deficiency, allowing increased resorption of bone by osteoclasts in many $src^{-/-}TRAPsrc^{K295M}$ mice (Schwartzberg

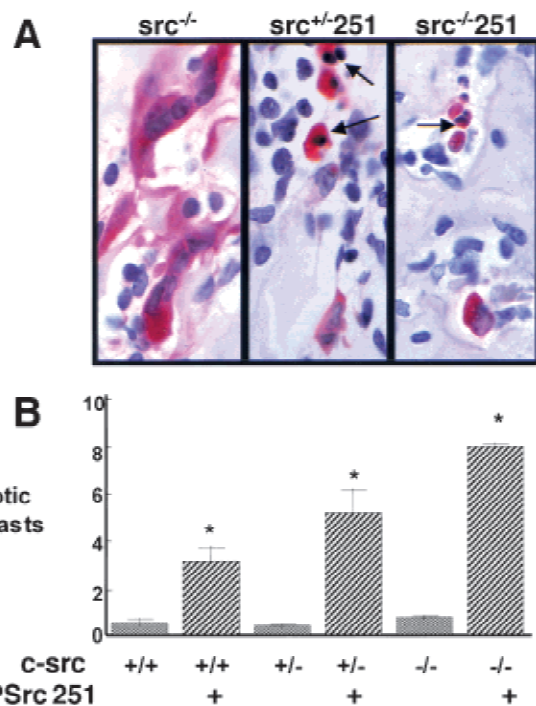**Figure 2.** *TRAPSrc^{251}* transgenic mice show increased osteoclast apoptosis. (*A*) Histological analyses, high power (100×), showing TRAP-positive osteoclasts. Osteoclasts in Src251 mice are intensely staining with pyknotic nuclei characteristic of apoptotic cells (arrows). (*B*) Percentage of osteoclasts that were apoptotic.

et al. 1997). Since Src251 differs from SrcK295M by the deletion of the kinase domain, one difference between these two molecules is the lack of regulation of the SH2 and SH3 protein interaction domains, which would not be masked by the kinase domain in Src251.

To examine whether misregulated domains of Src251 can affect Src function, we analyzed the subcellular partitioning of Src251 into detergent-soluble and insoluble fractions within osteoclasts. In unstimulated fibroblasts, wild-type Src is mostly in an inactive closed conformation that is detergent-soluble and associated with endosomes (Hamaguchi and Hanafusa 1987; Kaplan et al. 1992). Activation of Src is accompanied by translocation to a detergent-insoluble fraction (see Fig. 7C below; Clark and Brugge 1993; Walker et al. 1993; Kaplan et al. 1994); this subcellular change requires the protein interaction domains, but is independent of kinase activity, for inactivating the kinase by the K295M mutation does not prevent translocation. In contrast, Src251, with its available SH2 and SH3 domains, has been found to be constitutively detergent-insoluble in fibroblasts, where it is located in focal adhesions, the usual site of Src in an activated open conformation (Kaplan et al. 1994). Similarly, in osteoclasts, we found that Src251 resides predominantly in the detergent-insoluble fraction (Fig. 3A, lane 2). In osteoclasts, we also find that wild-type Src is more prevalent in the detergent-insoluble fraction, perhaps re-
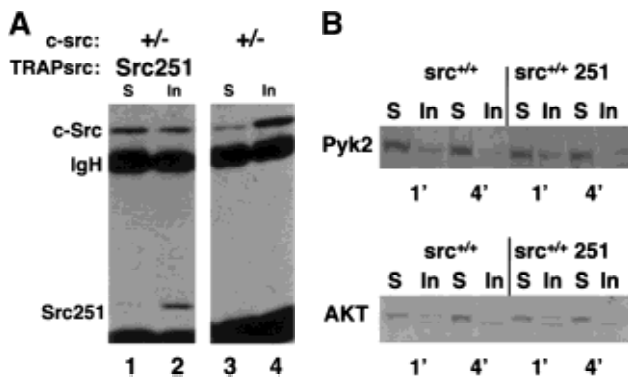
Figure 3. Src251 is detergent-insoluble in osteoclasts. (*A*) Osteoclasts were differentiated from bone marrow in culture, then lysed gently in 0.5% Triton X-100 for varying times (2-min lysis is shown here; similar results were obtained at other time points) and the remaining detergent-insoluble fraction then lysed with RIPA. Src was immunoprecipitated from detergent-soluble and -insoluble fractions and analyzed by immunoblotting (Kaplan et al. 1994). (*B*) Detergent solubility of Pyk2 and AKT are not altered by expression of Src251. Osteoclasts were lysed as above, and lysates were immunoblotted for Pyk2 and AKT. Similar results were seen at 1-, 2-, and 4-min detergent solubility lysis conditions (1- and 4-min conditions are shown here). (S) Detergent-soluble fraction; (In) detergent-insoluble fraction.

flecting its high expression and activity in this cell type (Fig. 3A, lane 4; Horne et al. 1992; Lowell et al. 1996). Significantly, however, when Src251 was expressed in osteoclasts, we observed a smaller proportion of the endogenous c-Src in the detergent-insoluble fraction than seen typically in wild-type osteoclasts (Fig. 3A, cf. lanes 2 and 4), suggesting that the presence of Src251 prevents wild-type Src from residing in the detergent-insoluble fraction. In contrast, we have found that expression of Src251 did not appear to alter significantly the kinase activity of c-Src in cultured osteoclasts (data not shown). Furthermore, Src251 did not appear to alter the subcellular localization of several other signaling molecules within the cell, including the tyrosine kinase Pyk2, the p85 subunit of Phosphatidylinositide 3-kinase (PI3K), and the serine-threonine kinase AKT (Fig. 3B; data not shown). These results suggest that Src251 may block cell-survival pathways by specifically competing with Src and perhaps other Src family kinases, preventing them from trafficking and interacting with other molecules upon activation.

Other Src mutants also induce apoptosis of osteoclasts

If misregulation of the protein interaction domains of Src251 causes apoptosis of osteoclasts in *Src251* transgenic mice, it might be expected that other Src mutants would also induce osteoclast apoptosis. In particular, high levels of expression of the *SrcK295M* mutant may not be properly regulated by Csk and, thus, might induce a similar phenotype. Although we did not see evidence of

increased osteoclast apoptosis in our initial analyses of *TRAPSrc^{K295M}* mice, overexpression of SrcK295M has been found to block signaling in a number of systems, including those activated by growth-factor receptors and G-protein-coupled receptors (Courtneidge et al. 1993; Twamley-Stein et al. 1993; Luttrell et al. 1996). To determine if increased levels of K295M also affect osteoclast survival, we examined bones from *TRAPSrc^{K295M}* transgenic mice that were bred to homozygosity to obtain higher levels of transgene expression. Surprisingly, we noted that the *TRAPSrc^{K295M/K295M}* homozygotes were less likely than hemizygotes to rescue the *src^{-/-}* phenotype (data not shown).

Significantly, we observed increased bone volume, more severe osteopetrosis, and increased osteoclast apoptosis in the nonrescued *src^{-/-}TRAPSrc^{K295M/K295M}* homozygous mice compared to *src^{-/-}* mice (Fig. 4A,B). Interestingly, when we compared levels of protein expression from the two series of transgenic mice, we found that the SrcK295M mutant is expressed at higher levels than Src251 (Fig. 4C). Thus, SrcK295M appears to be less potent, but may still act similarly to Src251 when expressed at high levels, perhaps because of misregulation of the protein interaction domains.

To further define the role of the SH2 and SH3 protein interaction domains in the induction of osteoclast apoptosis, we generated two additional transgenic lines that either deleted the region encoding the SH3 domain (*Src251-ΔSH3*) or changed a highly conserved arginine in the FLVRES sequence of the SH2 domain that is required for SH2 binding to phosphotyrosine residues (*Src251-R175L*) (Fig. 4D,E). The *TRAPSrc^{251-ΔSH3}* transgene also induced osteopetrosis that was associated with increased osteoclast apoptosis. However, mutation of the SH2 domain prevented the induction of apoptosis and osteopetrosis by Src251. Thus, induction of apoptosis by Src251 does not require the SH3 domain of Src251 but may require a functional SH2 domain.

Src251 affects AKT function

In several signaling pathways, protection from apoptosis involves the PI3K-mediated activation of the serine–threonine kinase AKT (Datta et al. 1997). Src family kinases have been implicated in the activation of PI3K through interactions with the PI3K p85 regulatory subunit (Fukui and Hanafusa 1991; Pleiman et al. 1994). Furthermore, pharmacological inhibition of PI3K activity can induce apoptosis of cultured osteoclasts (S. Kadapakkam and B.F. Boyce, unpubl.), suggesting that these signaling intermediates are important for osteoclast survival. To determine if Src251 may interfere with PI3K-mediated pathways, we examined AKT kinase activity in cultured osteoclasts. Osteoclasts cultured from Src251 mice had reduced AKT kinase activity compared to their wild-type counterparts (Fig. 5A). In contrast, the activation status of ERK1 and ERK2, other kinases involved in cell survival, did not appear to differ significantly between wild-type and Src251 cells (Fig. 5B).
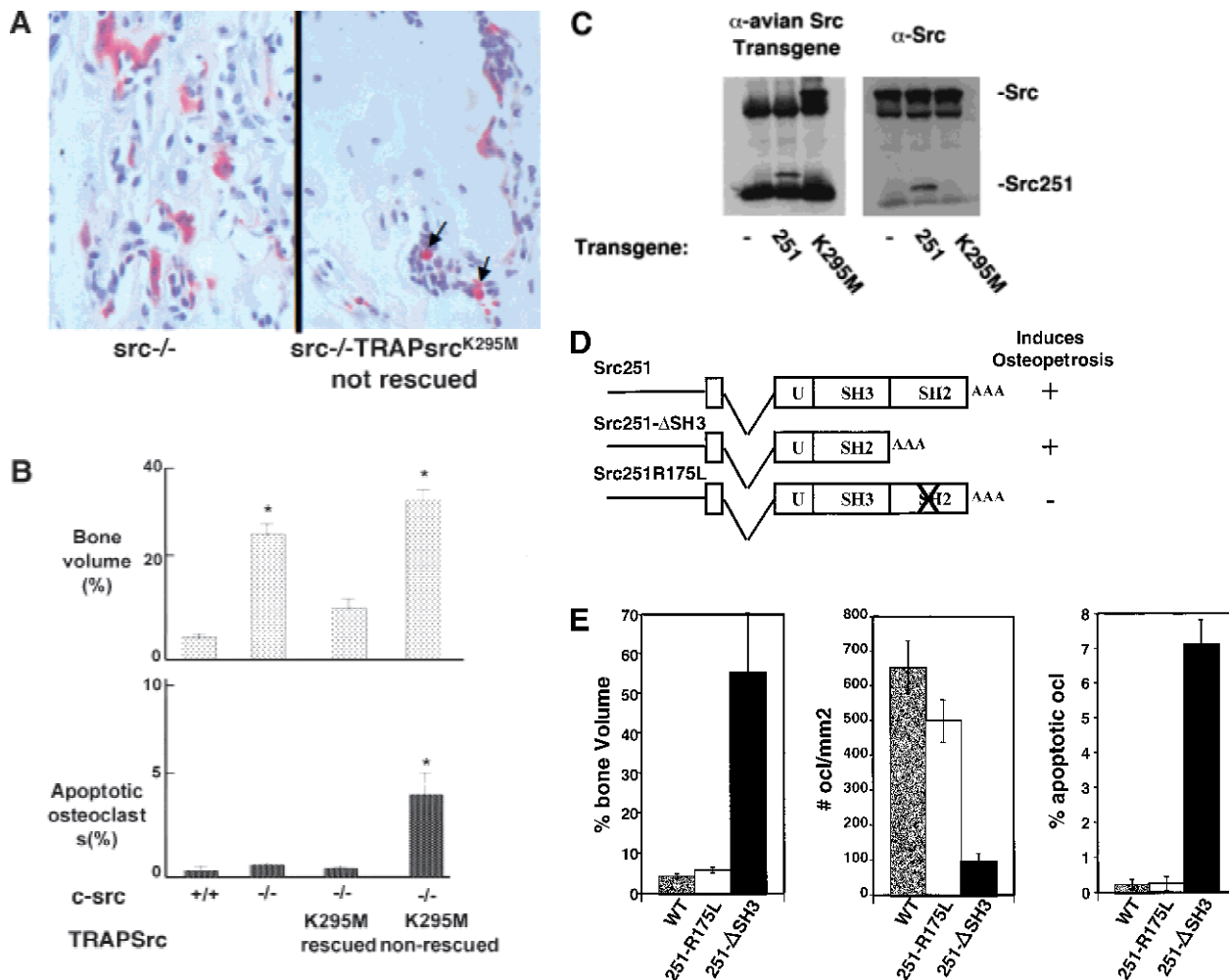
Figure 4. Other Src mutants induce apoptosis of osteoclasts. (*A*) Histological analyses of $src^{-/-}$ mice expressing the K295M kinase-inactive mutant that were not rescued. Arrows indicate apoptotic osteoclasts. (*B*) Bone volume and percentage apoptotic osteoclasts in wild-type (WT), $src^{-/-}$, and $src^{-/-}$ mice that showed rescue or did not show rescue by $TRAPSrc^{K295M}$. (*C*) Relative levels of expression of $Src251$ and $SrcK295M$ transgenes. Lysates from osteoclast cultures of Line V Src251 and Line NN SrcK295M transgenics were normalized for TRAP levels, Src-immunoprecipitated with monclonal 327, and immunoblotted with anti-avian specific Src monoclonal EC10, which only reacts with the transgenic Src (left panel), or monoclonal 327, (right panel) which reacts only with both endogenous and transgenic Src. Similar results were seen with other transgenic lines. (*D*) Phenotype of mutants affecting the SH3 and SH2 domains of Src251. (*E*) Bone histomorphometry, osteoclast numbers, and percent apoptotic osteoclasts from mutants affecting the SH3 and SH2 domains of Src251.

Thus, at least one pathway that may be altered by expression of $Src251$ is the activation of AKT.

$Src251$ decreases osteoclast survival in response to RANKL

Multiple pathways involved in cell survival have been shown to activate AKT, including those downstream from tyrosine kinase growth factor receptors, integrins, and TNF family receptors. Significantly, Src has been implicated in signaling from all these receptors (Thomas and Brugge 1997). In particular, recent data have suggested that Src is activated by the TNF family member RANKL via a TRAF6-mediated pathway that activates

AKT and may be involved in osteoclast survival (Wong et al. 1999a). Nonetheless, $src^{-/-}$ mice have high numbers of osteoclasts and do not appear to have decreased osteoclast survival in vivo. Indeed, we find that the percentage of apoptotic osteoclasts in bone sections from $src^{-/-}$ mice $(0.7\% \pm 0.1)$ is similar to that in wild-type mice $(0.5\% \pm 0.2)$, making the importance of Src in RANKL-mediated survival pathways unclear.

To examine whether Src251 interferes with this survival pathway in osteoclasts, we examined osteoclast survival in the presence or absence of RANKL. Wild-type osteoclasts rapidly lose viability in the absence of RANKL, whereas addition of RANKL promotes their survival (see Fig. 6A; Fuller et al. 1998). In contrast, osteoclasts cultured from Src251 mice failed to respond to
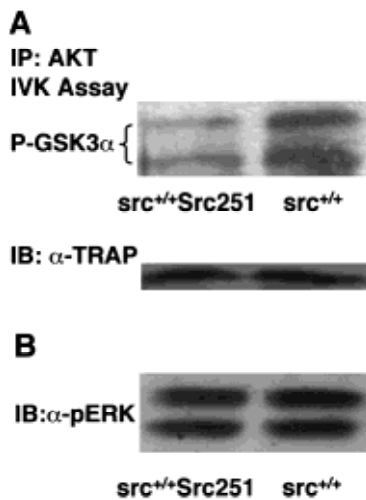
Figure 5. Src251 osteoclasts have reduced AKT kinase activity. (*A*) Osteoclasts were differentiated from bone marrow in culture, then lysed, immunoprecipitated with anti-AKT serum, and in vitro kinase assays were performed using GST–GSK3α as a substrate. Products were detected using an anti-phospho GSK-3 antibody by immunoblotting. After normalization for AKT protein levels, AKT activity was observed to be decreased twofold. In this experiment, twofold lower levels of AKT protein were also observed. (Lower panel) Lysates were normalized by immunoblotting for TRAP protein. (*B*) Osteoclasts were lysed as above and TRAP-normalized lysates were immunoblotted with anti-active ERK. Equivalent levels of total ERK were observed in these samples.

RANKL, with little or no increase in viability (Fig. 6A). Additionally, whereas we observe only slight increases in AKT activity in response to RANKL, these responses are reduced in osteoclasts from Src251 mice (Fig. 6B). Osteoclasts expressing the Src251-ΔSH3 mutant also failed to respond to RANKL, consistent with the ability of Src251-ΔSH3 to induce osteoclast apoptosis and osteopetrosis (Fig. 6C). Src251-R175L, in contrast, did not block osteoclast responses to RANKL, just as it did not induce osteopetrosis. Furthermore, as reported previously, $src^{-/-}$ osteoclasts also responded poorly to RANKL in culture (Fig. 6D; Wong et al. 1999a). The observation that Src deficiency prevents cell-survival responses to RANKL in vitro, as does expression of the *Src251* and *Src251-ΔSH3* mutants, strongly suggests that these mutants are blocking Src specific pathways in a dominant-negative manner. Thus, expression of these mutants may reveal a role for Src in RANKL-mediated cell survival in vivo that is not readily apparent from the phenotype of the Src-deficient mice alone.

Finally, another major pathway affecting osteoclast survival is that activated by the macrophage colony stimulating factor (M-CSF), which also rescues osteoclasts from apoptosis (Fuller et al. 1993). Experiments both in osteoclasts and in heterologous cells have implicated Src in M-CSF signaling (Courtneidge et al. 1993; Insogna et al. 1997). However, in contrast to the results with RANKL, $src^{-/-}$ osteoclasts and osteoclasts expressing Src251 were able to survive normally in response to

M-CSF in culture (Fig. 6D). Furthermore, AKT activity in response to M-CSF appeared normal in Src251 osteoclasts (Fig. 6E). Thus, the increased apoptosis in cells expressing *Src251* may result from a specific defect in response to RANKL.

Src251 can induce apoptosis in other cell types

To determine if the induction of apoptosis by Src251 may be a more generalized phenomenon shared with other cell types, we examined other cells that express this transgene. We have shown previously that virtually all mice carrying *TRAPSrc* transgenes express them in the intestine (Schwartzberg et al. 1997). Consistent with these patterns of expression, we found evidence of increased apoptosis in large-intestinal epithelial cells of *Src251* transgenic mice, suggesting that Src251 may inhibit cell-survival pathways in multiple cell types (Fig. 7A,B). Although we do not yet know the pathway(s) disrupted by Src251 in these cells, these results suggest that regulation of cell survival by Src-family kinases is not restricted to the osteoclast.

Discussion

Apoptosis, or programmed cell death, is an important regulatory process for many cells during embryonic development and normal function. For osteoclasts, as for many other hematopoietic cells, apoptosis is a normal part of their life cycle. The factors that contribute to osteoclast survival are therefore critical elements of bone homeostasis and development, which requires an active balance between the bone-generating activity of osteoblasts and the bone resorption of osteoclasts (Roodman 1999). Many factors have been found that influence osteoclast survival, including M-CSF and the recently discovered RANKL (Fuller et al. 1993, 1998). For example, estrogens induce osteoclast apoptosis via a TGF-β-mediated pathway that is associated with increased expression of the soluble decoy receptor osteoprotegerin, which binds and blocks RANKL activity (Hughes et al. 1996; Hofbauer et al. 2000). Thus, after menopause, as endogenous estrogens are reduced, not only is osteoclast formation increased, but osteoclast apoptosis is also decreased. The resulting increase in bone resorption is a major contributor to postmenopausal osteoporosis. Similarly, bisphosphonates, the first synthetic drugs found to reduce bone fractures in the elderly, increase osteoclast apoptosis (Hughes et al. 1995).

We demonstrate here that expression of a truncated form of Src with unregulated SH2 and SH3 protein interaction domains dramatically increases osteoclast apoptosis. Induction of apoptosis depends on a functional SH2 domain and is accompanied by decreased AKT activity and decreased responsiveness to RANKL. Recently, Wong and co-workers have implicated Src in RANKL-mediated activation of AKT via a TRAF6–Src interaction (Wong et al. 1999a). Our results suggest that Src family kinases are critical in vivo for the signal-
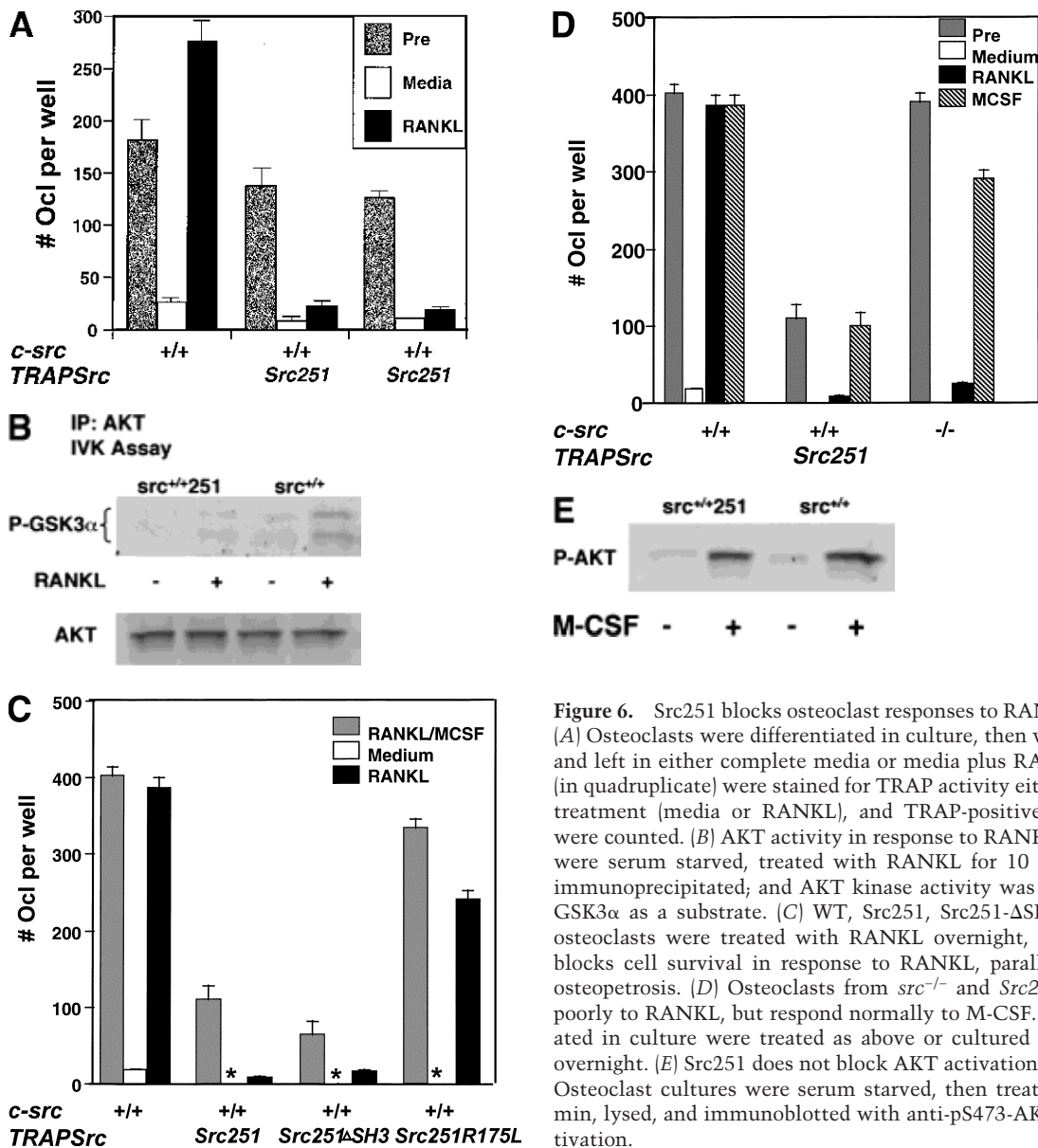
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Figure 6. Src251 blocks osteoclast responses to RANKL but not to M-CSF. (*A*) Osteoclasts were differentiated in culture, then washed twice with PBS and left in either complete media or media plus RANKL overnight. Wells (in quadruplicate) were stained for TRAP activity either before (pre) or after treatment (media or RANKL), and TRAP-positive multinucleated cells were counted. (*B*) AKT activity in response to RANKL. Osteoclast cultures were serum starved, treated with RANKL for 10 min, lysed, and AKT-immunoprecipitated; and AKT kinase activity was evaluated using GST–GSK3α as a substrate. (*C*) WT, Src251, Src251-ΔSH3, and Src251-R175L osteoclasts were treated with RANKL overnight, as in *A*. Src251-ΔSH3 blocks cell survival in response to RANKL, paralleling its induction of osteopetrosis. (*D*) Osteoclasts from $src^{-/-}$ and *Src251* mice both respond poorly to RANKL, but respond normally to M-CSF. Osteoclasts differentiated in culture were treated as above or cultured in media with M-CSF overnight. (*E*) Src251 does not block AKT activation in response to M-CSF. Osteoclast cultures were serum starved, then treated with M-CSF for 10 min, lysed, and immunoblotted with anti-pS473-AKT to monitor AKT activation.

ing pathways required for cell survival downstream from this TNF family receptor. Of note, although the in vitro data of Wong et al. implicated the Src SH3 domain in an interaction with TRAF6, our data indicate that this domain is not required for blocking this pathway. Rather, our data suggest that other parts of Src such as the SH2 and perhaps the unique domain may be critical for interactions with molecules in this pathway.

The implication of Src family kinases and tyrosine phosphorylation signaling in cell-survival pathways downstream from a TNF family receptor reveals a novel previously unappreciated cell survival signaling pathway from these receptors. Apoptosis has been associated with two distinct types of pathways that differ in their dependence on mitochondrial cytochrome C release and their ability to be blocked by expression of Bcl-2 family members. The availability of transgenic mice that express

BclXL in osteoclasts may allow further dissection of the RANK-induced signaling pathways involved in cell survival and the induction of apoptosis by Src251. Additionally, the use of Src mutants may permit further dissection of these cell-survival pathways.

Although RANKL is implicated in both osteoclast formation and survival, the effects of Src251 are most pronounced on osteoclast survival. It should be noted that the osteoclast survival experiments performed here used osteoclast cultures that had been differentiated in the presence of RANKL. Thus, neither Src deficiency nor expression of Src251 prevent osteoclast differentiation but, rather, they interfere specifically with osteoclast survival in response to RANKL. One interpretation of this data is that Src-family kinases may be less important for the signals downstream from RANK required for osteoclast formation. NFκB is also activated downstream
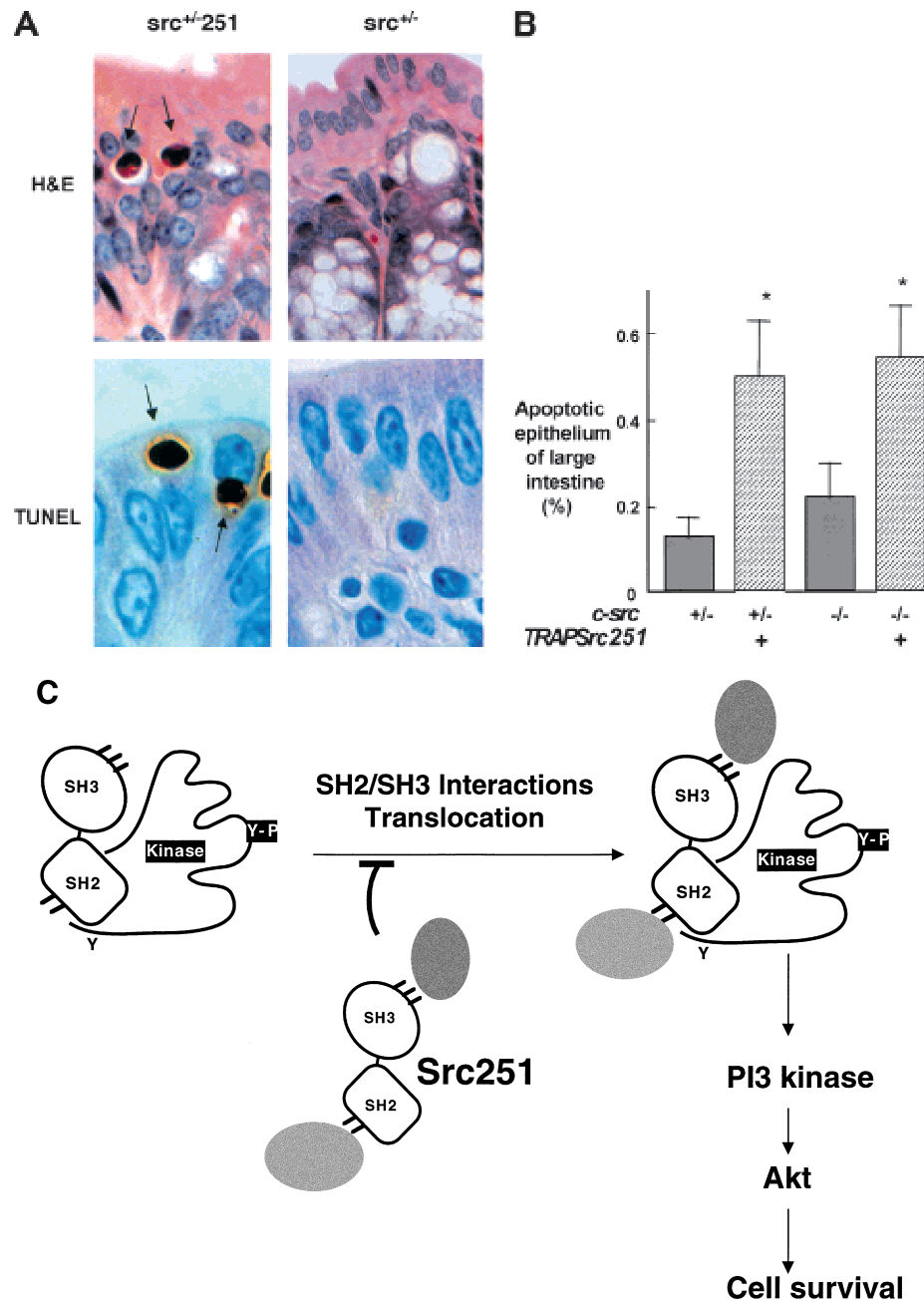
Figure 7. Src251 induces apoptosis of intestinal epithelial cells. (*A*) Histological analyses of large intestine (100×). Arrows indicate apoptotic epithelial cells. (Upper panels) H&E-stained sections; (lower panels) TUNEL staining showing apoptotic cells. (*B*) Percentage apoptotic large-intestine epithelial cells. Numbers were obtained from 3000 epithelial cells counted in each of two sections. (*C*) Model for action of Src251. Src251 is a particularly effective dominant-negative molecule because it efficiently blocks interactions of the Src homology protein interaction domains.

from RANK via TRAF6 (Wong et al. 1998). Combined deficiency of the NFκB p50 and p52 subunits or deficiency of RANK cause osteopetrosis as a result of defective osteoclast formation (Franzoso et al. 1997), indicating that distinct signaling pathways downstream from RANK may be involved in osteoclast formation and survival. Alternatively, it is possible that Src-family kinases may be involved in this NFκB activation pathway (Fig. 7D). Our results may then be secondary to relative

amounts of Src-family kinases expressed in preosteo-clasts and to the temporal patterns of expression of the Src251 transgene driven from the TRAP promoter, which may not be efficiently expressed in osteoclast precursors. RANKL has also been shown to activate osteoclasts to increase bone resorption (Fuller et al. 1998). Although it remains to be determined if defects in signaling from RANK contribute to the defects in bone resorption in $src^{-/-}$ osteoclasts, the recent report of osteo-

petrosis associated with defective ruffled borders in
$TRAF6^{-/-}$ mice (Lomaga et al. 1999), similar to the $src^{-/-}$
phenotype, suggests that this is likely. Indeed, our re-
sults raise the possibility that phenotypes associated
with deficiency of other Src-family kinases in mice may
result from defects associated with TNF-family receptor
signaling.

Although $src^{-/-}$ osteoclasts in culture also have re-
duced responses to RANKL in vitro, osteoclasts in Src-
deficient mice do not show increased apoptosis, suggest-
ing that other factors or pathways that are not present in
vitro can compensate for the lack of Src in vivo. These
could include other Src-family kinases or interacting
molecules with which Src251 may interfere. Indeed,
there is evidence that TRAF6 can coimmunoprecipitate
with multiple Src-family kinases (Wong et al. 1999a; A.
Venegas and P. Schwartzberg, unpubl.). Although non-
specific interactions or toxicity of Src251 cannot be ruled
out in these experiments, the degree of apoptosis in-
duced in osteoclasts appears to depend on a balance be-
tween the levels of endogenous Src and Src251. The ti-
tration of this phenotype against the level of endogenous
Src protein suggests that Src251 acts by specifically
blocking interactions of Src or other Src-family kinases
with other molecules. Thus, Src251 may inhibit cell-
survival pathways by specifically competing with Src,
perhaps by constitutively associating with proteins that
normally interact with Src and other Src-family kinases
(Fig. 7C). Indeed, preliminary experiments suggest that
although Src251 does not alter the subcellular localiza-
tion of Pyk2 and AKT, it may alter the detergent solu-
bility of TRAF6 (P. Schwartzberg, unpubl.), providing
further evidence for Src's connection to this signaling
pathway in osteoclasts.

The different phenotypes elicited from expression of
different *Src* mutants in the osteoclast, or even from the
same mutant (*K295M*) under different levels of expres-
sion, highlights the many roles for Src and Src-family
kinases in signal transduction. Although eliminating ki-
nase activity may not affect certain pathways such as
integrin signaling, it may severely impair others.

Our data with Src251 argue that inference with the
regulation of protein interaction domains may be a par-
ticularly effective means of blocking Src-family function
and again emphasize the importance of these domains in
Src function. Expression of Src251 can also act as a po-
tent block to VEGF-induced angiogenesis in a chicken
chorioallantoic membrane model system (Eliceiri et al.
1999) and can induce apoptosis in intestinal epithelial
cells (Fig. 7A,B). Thus, as in the osteoclast, expression of
the dominant-negative Src251 may reveal roles for Src-
family kinases in pathways that are not obvious from the
gross phenotype of $src^{-/-}$ mice.

It is therefore important to note that whereas our re-
sults highlight the potential of Src inhibition as a drug-
targeting strategy, they also underscore the potential
hazards of Src inhibition that may not be discerned in
studies using isolated tissue culture systems. Osteo-
clasts provide a model system for understanding Src
function in a physiologic setting and help provide infor-

mation for therapeutic development based on Src family
members. In bone, this may be particularly useful for the
treatment both of osteoporosis and of rheumatoid arthri-
tis, where blocking RANKL activity has been found to
decrease bone destruction in animal models (Kong et al.
1999a). However, such therapeutics may also be useful
for cancers and other pathophysiologic conditions in
which Src or other Src-family kinases have been impli-
cated.

Material and methods

Transgenic mice

Generation of the $TRAPSrc^{WT}$ and $TRAPSrc^{K295M}$ transgenic
constucts and transgenic mice were described previously.
$TRAPSrc^{251}$ was constructed by PCR amplification of the se-
quence encoding the first 251 amino acids of the chicken c-*src*
gene, and a *Bst*Y1–*Xba*1 fragment was used to replace a portion
of the wild-type chicken *src* gene in pBS*TRAPSrc*. To generate
$TRAPSrc^{251-R175L}$, $TRAPSrc^{251}$ was mutagenized using the
Quickchange kit (Stratagene) using the following primer and its
reverse complement: (GGA ACC TTC TTG GTC CTC GAG
AGC GAG ACG). To generate $TRAPSrc^{251-\Delta SH3}$, a fragment was
subcloned into $TRAPSrc^{251}$ from $Src251-D11$ (Kaplan et al.
1994). Transgenic mice were generated via standard technology.
Transgenic founders were bred to $src^{+/-}$ mice and then interbred
to generate *TRAPSrc* transgenic mice in wild type, $src^{+/-}$, and
$src^{-/-}$ backgrounds. Genotyping was performed as described pre-
viously (Schwartzberg et al. 1997).

Antibodies and reagents

The following antibodies were used in this study: Monoclonal
anti-c-Src 327 (Oncogene Science and gift of Joan Brugge, Har-
vard Medical School, Boston, MA); Src2 polyclonal, Pyk2 poly-
clonal, p85 PI3K polyclonal, and anti-active ERK monoclonal E4
(Santa Cruz), EC10 anti-chicken Src specific monoclonal (UBI),
anti-TRAcP monoclonal (Zymed), and anti-GSK3 polyclonal,
anti-AKT polyclonal, and anti-pS473-AKT polyclonal (New En-
gland Biolabs). RANKL was a generous gift from Dr. Dirk
Anderson (Immunex Corporation, Seatttle, WA) and Dr. Mehr-
dad Tondravi (Redcross, Rockville, MD).

Histologic and radiographic analyses

Preparation and analyses of bones were performed as described
previously (Schwartzberg et al. 1997). Briefly, bones were fixed
in 10% buffered formalin, decalcified in 10% EDTA, and em-
bedded in Paraffin. Sections were stained with Hematoxylin and
Eosin and for TRAP activity. Bone volume (amount of bone
matrix as a percentage of cancellous space) and osteoclast num-
bers (per millimeter of the cancellous bone surface) in the can-
cellous space of the long bones were measured as described
previously (Boyce et al. 1992). For histology, samples were ex-
amined from a minimum of four mice each. Apoptotic osteo-
clasts were determined by TRAP staining or TUNEL analyses
(Hughes et al. 1996). The assessment of colonic enterocytes
apoptosis was done in the upper half of the crypts of sections of
the ascending colon. Three thousand enterocytes were counted
in each of two sections, and apoptotic cells were identified using
standard morphologic criteria on H&E stained sections. Radio-
graphs were performed on a Faxatron X-ray machine.

Osteoclast cultures

Osteoclasts were differentiated from bone marrow in the pres-
ence of Vitamin D3 (10^{-9} M) with or without Dexamethasone (5

nm/mL) as described previously (Takahashi et al. 1988b). Alternatively, bone marrow and splenocytes were cultured in the presence of osteoblasts derived from calvaria from newborn mice (Takahashi et al. 1988a). Cultures were enriched for osteoclasts by removing osteoblast feeder layers by rinsing with phospho buffered saline (PBS) without Ca^{2+} and Mg^{2+}.

For cell-survival studies, osteoclasts were differentiated from CFU-GM cultures (Uy et al. 1995). Briefly, splenocytes were cultured in methylcellulose in the presence of recombinant human GM-CSF (20 ng/mL) and M-CSF (30 ng/mL) for 11–14 d. Pools of five colonies were plated in α-MEM with 10% fetal calf serum, glutamine, penicillin, and streptomycin in the presence of 80 ng/mL RANKL and 30 ng/mL M-CSF. Under these conditions, osteoclasts had differentiated by 3–5 d.

Survival assays

Differentiated osteoclasts were washed three times with PBS, then either stained for TRAP activity or incubated overnight in complete α-MEM alone or supplemented with 100 ng/mL RANKL or 30 ng/mL M-CSF. After 24 h, cells were stained for TRAP activity. TRAP-positive cells containing three or more nuclei were scored as osteoclasts.

Immunoprecipitations and Western analyses

For detergent-insolubility assays, osteoclasts differentiated in six well plates were lysed by gently pipetting 400 µL of 0.5% Triton X-100 buffer for varying times. Soluble material was removed by pipetting, then cells were further lysed to obtain detergent-insoluble material by the addition of RIPA (Radio-immunoprecipitation buffer: 50 mM Hepes at pH 7.0, 1% Triton X-100, 1 mM EDTA, 0.1% sodium dodecyl sulfate, 1% sodium deoxycholate, 150 mM NaCl with protease inhibitors and sodium orthovanadate) for 10 min at 4°C (Kaplan et al. 1994). Detergent-soluble and -insoluble fractions were normalized by addition of the opposite lysis buffer, and lysates were clarified by centrifugation at 14,000g for 10 min. Src was immunoprecipitated from cell lysates using anti-Src monoclonal 327; immunoprecipitates were resolved on a 10% SDS–polyacrylamide gel and immunoblotted with a combination of EC10 and Src-2 antibodies. Alternatively, lysates were resolved by SDS-PAGE and immunoblotted for Pyk2, p85, or AKT.

To evaluate transgenic protein levels, osteoclast cultures were lysed in RIPA, Src-immunoprecipitated with monoclonal 327, and immunoblotted with either monoclonal EC10, which only recognizes the chicken c-Src transgenic protein, or 327, which recognizes both endogenous and transgenic Src.

AKT kinase assays

Osteoclasts were differentiated from bone marrow as above, osteoblasts were washed away with PBS, and the cells were lysed in 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X-100, 2.5 mM sodium pyrophosphate, 1 mM beta glycerophosphate, 1 mM sodium orthovanadate, 1 µg/mL leupeptin, 1 mM PMSF, plus Protease Inhibitor Mini-Tablet/10 mL (Roche Molecular Biochemicals). AKT was immunoprecipitated, and kinase activity was assayed with the New England Biolabs AKT kinase assay kit (NEB 9840) using GST–GSK-3α as a substrate. Products were detected using an anti-phospho-GSK3 antibody as per the manufacturer's recommendation.

To examine responses to RANKL or M-CSF, differentiated osteoclast cultures were serum starved in 0.5% serum for 6 h, then treated with RANKL (1 µg/mL) or M-CSF (300 ng/mL) for 10 min and lysed as above. Lysates were resolved by SDS-PAGE and immunoblotted with anti-pS473-AKT or anti-AKT sera for M-CSF stimulated cultures, or analyzed for AKT kinase activity as above (RANKL-treated cultures).

Acknowledgments

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Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival

Lianping Xing, Ana M. Venegas, Amy Chen, et al.

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