Cell, Vol. 77, 795–798, June 17, 1994, Copyright © 1994 by Cell Press

NF-AT–AP-1 and Rel–bZIP: Hybrid Vigor and Binding under the Influence

Minireview

Garry P. Nolan
Department of Molecular Pharmacology
Stanford University School of Medicine
Stanford, California 94305

During the evolution of transcriptional control of immediate-early immune system responses, the Rel DNA-binding and dimerization domain has brokered its way into many an influential family. The recent cloning by two groups (McCaffrey et al., 1993a; Northrop et al., 1994; Figure 1) of different Rel-containing cytoplasmic components of the nuclear factor of activated T cells (NF-AT) (Shaw et al., 1988) exemplifies this with a physiologically relevant association of the Rel domain beyond the ankyrin (IκB/Bcl-3) proteins to include polypeptides of the basic–leucine zipper (bZIP) families. Biochemical dissection of the NF-AT complex into both Rel- and AP-1-like protein subcomponents solidifies earlier work proposing that intracellular signaling leads to the formation of DNA-bound Rel–bZIP transcription factor supracomplexes (TFSs) with physiologic functions and DNA-binding capacities distinct from that of either transcription factor partner (LeClair et al., 1992; Stein et al., 1993a, 1993b).

NF-AT points to a model in which the regulatory circuitry within T cells can integrate signals at genes by facilitating the binding and assembly of mixed-family (Rel–bZIP) TFSs to novel DNA motifs. Here I show that elucidation of the molecular nature of the NF-AT subcomponents provides important links between well-described Rel polypeptides and other transcription factor signaling pathways with cells.

A Role for AP-1 in NF-AT Assembly

The story begins with events proceeding from T cell antigen receptor engagement. Antigen-dependent activation of T cells in an immune response leads to the immediate-early expression and secretion of the cytokine interleukin-2 (IL-2), surface expression of the IL-2 receptor complex, and eventual commitment of the T cell to a determined CD4- or CD8-specific function. Signaling events post-CD3 activation and phospholipase C-γ1 lead to two separate signals: phosphokinase C (PKC) pathway activation and release of intracellular Ca^{2+} stores with a subsequent extracellular Ca^{2+} influx (Figure 2A). These two signals can be mimicked by treatment of T cells with the pharmacologic agents phorbol myristate acetate and ionomycin, a PKC agonist and a Ca^{2+} ionophore, respectively (Figure 2A).

These two signals lead to the immediate-early expression of IL-2 and the correlated, rapid appearance of the protein NF-AT (within 30 min) that binds to two sites in a 300 bp enhancer region upstream of the transcriptional initiation site of IL-2 (Shaw et al., 1988). NF-AT is composed of, in part, a preexisting cytoplasmic component that translocates upon T cell activation into the nucleus, first designated NF-AT_c (Flanagan et al., 1991) for cytoplasmic and, alternately, NF-AT_p for preexisting (McCaffrey et al., 1993b). NF-$AT_{p/c}$ is a target of the Ca^{2+} arm of the T cell activation pathway as agents acting here can either induce NF-$AT_{p/c}$ translocation (by constitutive expression of calcineurin serine/threonine phosphatase activity) or inhibit NF-$AT_{p/c}$ translocation into the nucleus (by application of the clinically important immunosuppresants CsA or FK506 that activate immunophilins to inhibit NF-AT_c activation) (Bram et al., 1993; Flanagan et al., 1991; Clipstone and Crabtree, 1992; McCaffrey et al., 1993b). Within the nucleus, NF-$AT_{p/c}$ can combine with a ubiquitous, newly synthesized nuclear component to form a DNA-binding supracomplex termed NF-AT (Figure 2A).

The ubiquitous, induced nuclear component is an AP-1-like complex, comprised at least in part by Fos or Jun (Jain et al., 1992). The identity of the induced nuclear component was suggested by the PKC activation requirements needed for assembly of a holo-NF-AT complex and blockage of its assembly by protein translation inhibitors. Consistent with the activation requirements for Fos/Jun and related polypeptides, the appearance of the nuclear subcomponent is not blocked by CsA or FK506. Finally, AP-1 competitor oligonucleotides specifically inhibit the binding of NF-AT to its cognate motif, even though the NF-AT-binding site does not contain a recognizable AP-1 site. Interestingly, antibody supershifting experiments using anti-Jun or anti-Fos supershift only a part of the complex, perhaps indicating that the nuclear subcomponent

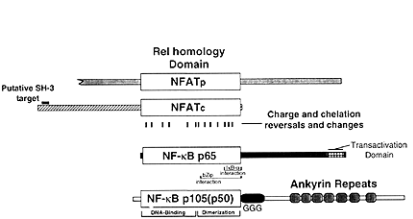

Figure 1. The Human and Murine NF-AT Rel Clones Aligned with NF-κB Component p65 and the p50 Precursor p105

Charge reversals of NF-AT Rel proteins compared with NF-κB Rel are shown. The horizontal bar at the amino terminus of the human NF-AT Rel is a putative target for SH-3 domains. The amino terminus of the NF-AT_c clone is shown as a broken bar to depict that the current murine cDNA clone is not full length.

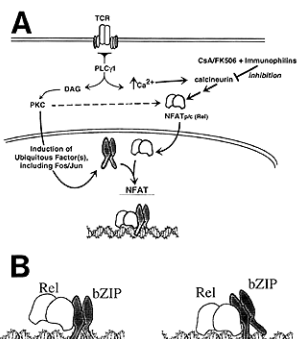

Figure 2. Models for Associative Binding of bZIP Proteins to Rel Proteins, Including NF-AT$_{pc}$–AP-1

(A) Signaling pathways leading to the assembly of the NF-AT supracomplex on DNA. The preexisting cytoplasmic precursor of NF-AT (NF-AT$_{pc}$) is shown as an inducible dimer as speculation owing to its homology to Rel proteins. The ubiquitous factor is shown as a Fos/Jun dimer owing to data suggesting this identity. The effect of PKC activity upon NF-AT$_c$ phosphorylation (Northrop et al., 1994) is shown as a broken arrow.

(B) Models for Rel–bZIP-stabilized binding leading to the formation of TFSs. Depicted are strong binding by bZIP or Rel proteins stabilizing a weak Rel–DNA or bZIP–DNA interaction, respectively. Not shown are individually weak or strong Rel–DNA and bZIP–DNA interactions cumulatively leading to a stable (Rel–bZIP)–DNA interaction.

is represented by a heterogeneous population of AP-1/ZIP polypeptides (Boise et al., 1993; Jain et al., 1992).

AP-1 participation in the NF-AT complex contributes to high affinity DNA binding of the NF-AT$_{pc}$ subcomponent. For instance, two NF-AT complexes are observed binding to the murine IL-2 NF-AT DNA motif (gcccAAaGAGGAAAAttTGTTTCATACAG; identities to the human sequence in bold): an upper complex consisting of an NF-AT$_p$–AP-1 supracomplex and a second, faster-migrating complex containing only NF-AT$_p$ (Jain et al., 1992, 1993a, 1993b). The upper (NF-AT$_p$–AP-1)–DNA complex has a longer half-life than the NF-AT$_p$–DNA complex, suggesting that the presence of AP-1 confers DNA binding stability on NF-AT$_p$. In contrast, only the NF-AT$_p$–AP-1 complex is formed on the human motif (aagaAAgGAGGAAAAacTGTTTCATACAG; identities to murine sequence in bold). The critical difference lies not in the species source of the proteins but in the sequence to which NF-AT$_p$ binds. The ac replacement in the human motif results in a selective inability to form either the human or murine NF-AT$_p$ lower complex (Jain et al., 1993b). Thus, NF-AT$_p$ binding to the human DNA motif is made possible in vitro by assembly of an NF-AT$_p$–AP-1 supracomplex.

This is interesting since part of the murine IL-2 NF-AT motif, underlined above, shows respectable similarity to Rel protein-binding motifs of NF-κB Rel proteins based on the expanded κB consensus GRGRNNYYYY and known binding sites for Drosophila Rel proteins dorsal (GGGTTTCTCC) and Dif (GGGGATTTTT) (Ip et al., 1993). Mutation of the murine terminal TT residues (GAGGAAAATT) to the AC sequence found in the human IL-2 NF-AT motif (GAGGAAAAAC) lowers the similarity of the site to the DNA-binding consensus for known Rel proteins and is correlated with a loss of DNA complex corresponding to the lone NF-AT$_p$ component. The recognition that NF-AT$_p$ and NF-AT$_c$ are Rel domain proteins implies that AP-1 or other proteins might have a general role in determining binding stability or specificity of Rel proteins.

The Preexisting and Cytoplasmic Rel Components of NF-AT

Biochemical characterization of the binding requirements for NF-AT made possible the cloning of two different genes encoding the preexisting and the cytoplasmic components of NF-AT. Murine cDNAs for NF-AT$_p$ were cloned according to their assayed ability to form a DNA-binding complex with a murine NF-AT site without the contribution of AP-1-like cofactor binding (McCaffrey et al., 1993a). Human and murine NF-AT$_c$s were cloned by assaying column fractions from thymus for a cytoplasmic factor that can bind to the human NF-AT motif in association with an ubiquitous nuclear component from HeLa nuclear extract (Northrop et al., 1994). The differences in cloning strategy might help to explain why the cDNAs isolated in the murine and human systems are similar, yet not necessarily functional analogs (see Figure 1).

The salient unifying feature of the predicted proteins is that both contain a Rel homology region of ~270 amino acid residues (see Figure 1). Strikingly, the Rel domain is at the carboxyl terminus of NF-AT$_c$ and in the middle of NF-AT$_p$ (Northrop et al., 1994; McCaffrey et al., 1993a). Rel homology domains in the NF-κB proteins all contain their Rel domain in the amino-terminal portion of the molecule (Nolan and Baltimore, 1992). The Rel domains of NF-AT$_{pc}$ are distantly related to the NF-κB family of Rels, showing a maximal 20% cumulative identity to the Rel domains of p50, p65, and c-Rel, with the most homology found in the DNA-binding two thirds. The Rel domains of NF-κB polypeptides display between 50% and 60% identity to each other (Nolan et al., 1991). NF-AT$_p$ and NF-AT$_c$ demonstrate >70% identity over the recognizable Rel domain with no need for introduced gaps. Outside the Rel domain, however, NF-AT$_c$ and NF-AT$_p$ each encode extensive amino-terminal domains that show minimal similarity to each other or to anything else in the data bases, but contain a preponderance of proline and serine residues. The proline residues are consistent with a role in transcriptional activation, and serine residues might be a target for calcineurin, the serine/threonine phosphatase known to be active in the NF-AT pathway. Of the cDNAs isolated to date, human NF-AT$_c$ shows amino-terminal splicing variation, whereas murine NF-AT$_p$ demonstrates

carboxy-terminal alternative splicing (McCaffrey et al., 1993a; Northrop et al., 1994).

Alignment of NF-AT Rel to NF-κB Rel domains shows there has been a striking global switch of charged residues in the NF-AT Rel domains as compared with the known NF-κB-related Rel domains (see Figure 1). At several positions all NF-κB Rel domains conserve a positively charged residue, such as lysine or arginine that NF-AT Rel has changed to aspartate or glutamate (the converse is also observed). Similarly, there are changes in the NF-AT Rel domain at residues that might be important for DNA binding, zinc chelation (Liou and Baltimore, 1993), or salt bridge formation. If one wanted to design an NF-AT Rel domain surface that could not interact with the Rel domains of the NF-κB proteins (p50, p65, c-Rel, p52, or RelB), a strategy might be to switch the corresponding charge residues on either face of the interacting surfaces. Given the distinct role of NF-AT in T cell development and the role of NF-κB proteins in the activation of other distinct immune and nonimmune system genes, it would not be surprising that different subgroups of Rel proteins have evolved strategies to minimize signaling pathway cross-talk. It remains to be determined whether NF-AT Rel domains can dimerize with NF-κB Rel domains or associate with IκB ankyrin-like polypeptides.

Recombinant proteins derived from the respective murine and human NF-AT Rel-containing cDNAs reconstitute the expected biochemical, DNA binding, and transcriptional activities. Transfection of full-length human NF-AT_c leads the expression of the predicted 115 kDa protein by Western blotting; transfection of this subunit circumvents the need for a calcium signal in activation of NF-AT-dependent reporter genes in T cells, suggesting an override of a negative regulatory mechanism upon NF-AT Rel due to overexpression (Northrop et al., 1994). Immunoblotting demonstrates increased mobility of the transfected protein in SDS–polyacrylamide gels after ionomycin treatment, inhibitable by CsA, suggesting phosphorylation control of NF-AT Rel (Northrop et al., 1994). Truncated recombinant murine NF-AT_p, requiring the complete Rel domain, binds to its cognate murine NF-AT sites with specificity and associates only with bZIP-containing Jun and Jun/Fos recombinant proteins to form a slower-migrating complex. In in vitro transcription assays, this truncated protein is transcriptionally inactive but allows for potent activation through NF-AT motifs in the presence of transcriptionally active forms of Jun/Fos or Jun dimers containing the bZIP motif (McCaffrey et al., 1993a).

Importantly, the NF-AT_c and NF-AT_p mRNAs have distinct tissue distributions and induction characteristics. NF-AT_c expression appears largely restricted to T cells, correlating with prior results for NF-AT protein expression in spleen and thymus, with no apparent expression in B cell lines (Northrop et al., 1994). In contrast, human NF-AT_p message appears to be expressed beyond T cells and can also be found in brain, heart, and testis. An important distinction is that NF-AT_c is inducible by phorbol myristate acetate and ionomycin in T cells, whereas NF-AT_p is not. Therefore, the relative role played by NF-AT_c–AP-1 versus that of NF-AT_p–AP-1 (with the potential for NF-AT_c/NF-AT_p dimers or interplay with other bZIP proteins) during early to late gene expression in T cells and beyond is likely to be a rich arena for subsequent investigation.

Rel–bZIP Associations and a TFS–DNA Dialectic

Since AP-1-like complexes can contribute to the regulatory capacity of the NF-AT Rel protein, it is notable that NF-κB Rel proteins and Fos/Jun have recently been demonstrated to interact in vitro (Stein et al., 1993a). Starting with an observation that transfection of Fos or Jun could activate the HIV-1 long terminal repeat in a κB-dependent manner, Stein et al. (1993a) narrowed the mechanism to a strong, specific interaction of Fos or Jun with the p65 subunit of NF-κB. The interaction was dependent on a region in the carboxyl half of the Rel domain of p65 and required a functional ZIP region in Fos or Jun. The Fos/Jun activation could not be substituted for by JunB or JunD, suggesting a specific interaction with only certain members of this family. Transcriptional synergy through a κB site in a reporter gene paralleled the in vitro results in the requirement for p65, a functional ZIP in Fos or Jun and the bZIP activation domain of Fos or Jun. The data suggest these NF-κB–Fos/Jun complexes might not have been recognized as such because their migration in standard electrophoretic mobility shift assays appears similar to that of cytoplasmic NF-κB. Reminiscent of the NF-AT Rel/AP-1 stabilization, antibody supershifting evidence suggests that a role of Fos/Jun at certain κB sites might be to stabilize the binding of the Rel components to their cognate site while not participating directly in the gel shift complex, perhaps becoming dissociated during electrophoretic separation (Stein et al., 1993a).

The C/EBP family of proteins, which also contain a bZIP region, describe a remarkably similar story. Early interaction–cloning experiments using recombinant NF-κB p50 protein as a probe allowed for the isolation of an interesting cDNA clone: C/EBPβ (LeClair et al., 1992). Thematically, the interaction required the Rel domain of p50 and the ZIP structure of C/EBPβ. Although it was not possible in these experiments to observe a DNA-bound supracomplex of C/EBP and p50, a consistent augmentation of p50 binding (stabilization) to the κB motif was observed. This again suggests a DNA-binding facilitation/stabilization step during interaction of bZIP proteins and Rel proteins, seen previously with AP-1–NF-κB Rel and AP-1–NF-AT_p Rel. Building on these studies, the NF-κB family members p50, p65, and c-Rel, along with C/EBP proteins α, β, and δ, show a similar physical interaction that is dependent upon their respective Rel and ZIP domains (Stein et al., 1993b). This interaction can also be observed in cells through potent synergistic transcriptional activation through the C/EBP-binding motif, the serum response element. Surprisingly, κB motif–dependent expression is blocked by the cotransfection of NF-κB subunits and C/EBP proteins, suggesting that distinct positive or negative roles during cross-coupling might be dependent upon the presence or absence of a cobinding cognate site for each transcription factor.

It is clear then that bZIP-containing proteins of the C/EBP family and certain members of the AP-1 family of complexes, such as Fos/Jun, are capable of specific inter-

actions with Rel proteins of diverse evolutionary heritage. Are there conserved residues in the Rel and bZIP domains that mediate such interactions? Such Rel–bZIP cross-coupling could induce local DNA conformational changes important for gene regulation or might serve to stabilize otherwise weak interactions between DNA and individual Rel or bZIP proteins (see Figure 2B). Thus, such protein associations might effectively broaden the DNA motifs potentially bound by a given "individual" transcription factor. And, as corrolary, the binding of a Rel protein to its cognate motif might draw in bZIP proteins to act as cofactors to bind at DNA regions they would not necessarily bind in the absence of the Rel polypeptides. As an aside, such associations might also be a source in evolution for non-DNA-binding transcriptional coactivators resulting from the evolved loss of DNA-binding domains from a previous DNA-binding transcription factor partner.

Similarly, a model can be proposed in which Fos/Jun (bZIP) would provide much of the DNA binding capacity and Rel components would contribute their transcriptional activation domains (Figure 2B, left), as suggested in co-transfection of a TPA-reponsive element–dependent reporter gene with p65 and Fos or Jun subunits (Stein et al., 1993a). Such an important change in how one correlates DNA-binding complexes to transcriptional activation could force a reanalysis of the the transcription factor activation domains that act upon the proteins assembling at the TATA and initiator boxes. Indeed, the combined contributory affinity of multiple individual transcription factors acting in a supracomplex might allow for TFS binding to highly degenerate (and perhaps to us unrecognizable) DNA motifs, as appears the case for the binding of NF-AT to the human NF-AT site. This latter point is uncertain ground for investigators wishing to identify and discern how the components of such supracomplexes contribute to control of gene action.

Conclusion

The contribution of NF-AT to T cell commitment gives biological substance to interactions between Rel and bZIP proteins in the induced association of a preexisting cytoplasmic Rel protein and AP-1-like factors to create a TFS with unique activities. Recent findings suggest that the induction of Rel–bZIP interactions might also have relevance to infection and disease progression, as evidenced by the Epstein–Barr virus bZIP protein BZLF1 and interaction with p65 in B cells (Gutsch et al., 1994). Notably, cross-coupling is not unprecedented for the AP-1-related family of factors, as this group of bZIP proteins seems to have made an evolutionary habit of sticking their domains into other transcription factor families' business, as seen by interactions between Fos/Jun and glucocorticoid proteins or Jun and the basic–helix-loop-helix protein MyoD (Bengal et al., 1992; Jonat et al., 1990; Diamond et al., 1990). Given the close quarters during mRNA initiation and the number of proteins that manage to get involved, it seems satisfactory, then, that the Rel and bZIP polypeptides have evolved the capacity to manage these interfamilial affairs profitably.

References

Bengal, E., Ransome L., Scharfmann, R., Dwarski, V. J., Tapscott, S. J., Weintraub, H., and Verma, I. (1992). Cell *68*, 507–519.

Bram, R. J., Hung, D. T., Martin, P. K., Schreiber, S. L., and Crabtree, G. R. (1993). Mol. Cell. Biol. *13*, 4760–4769.

Clipstone, N. A., and Crabtree, G. R. (1992). Nature *357*, 695–697.

Diamond, M. I., Miner, J. N., Yoshinaga, S. K., and Yamamoto, K. R. (1990). Science *249*, 1266–1272.

Flanagan, M. W., Corthesy, B., Bram, R. J., and Crabtree, G. R. (1991). Nature *352*, 803–807.

Gutsch, D. E., Holley-Guthrie, E. A., Zhang, Q., Stein, B., Blanar, M. A., Baldwin, A. S., and Kenney, S. C. (1994). Mol. Cell. Biol. *14*, 1939–1948.

Ip, Y. T., Reach, M., Engstrom, Y., Kadalayil, L., Cai, H., Gonzalez, S., Tatei, K., and Levine, M. (1993). Cell *75*, 753–763.

Jain, J., McCaffrey, P. G., Valge-Archer, V. E., and Rao, A. (1992). Nature *356*, 801–804.

Jain, J., McCaffrey, P. G., Miner, Z., Kerpolla, T. K., Lambert, J. N., Verdine, G. L., Curran, T., and Rao, A. (1993a). Nature *365*, 352–355.

Jain, J., Miner, Z., and Rao, A. (1993b). J. Immunol. *151*, 837–848.

Jonat, C., Rahmsdorf, H. J., Park, K. K., Cato, A. C. B., Gebel, S., Ponta, H., and Herrlich, P. (1990). Cell *62*, 1189–1204.

LeClair, K., Blanar, M. A., and Sharp, P. A. (1992). Proc. Natl. Acad. Sci. USA *89*, 8145–8149.

Liou, H.-C., and Baltimore, D. (1993). Curr. Opin. Cell Biol. *5*, 477–487.

McCaffrey, P. G., Luo, C., Kerppola, T. K., Jain, J., Badalian, T. M., Ho, A. M., Burgeon, E., Lane, W. S., Lambert, J. N., Curran, T., Verdine, G. L., Rao, A., and Hogan, P. G. (1993a). Science *262*, 750–754.

McCaffrey, P. G., Perrino, B. A., Soderling, T. R., and Rao, A. (1993b). J. Biol. Chem. *268*, 3747–3752.

Nolan, G. P., and Baltimore, D. (1992). Curr. Opin. Genet. Dev. *2*, 211–220.

Nolan, G. P., Ghosh, S., Liou, H.-C., Tempst, P., and Baltimore, D. (1991). Cell *64*, 961–969.

Northrop, J. P., Ho, S. N., Thomas, D. J., Chen, L., Timmerman, L., Nolan, G. P., Admon, A., and Crabtree, G. R. (1994). Nature, in press.

Shaw, J., Utz, P. J., Durand, D. B., Toole, J. J., Emmel, E. A., and Crabtree, G. R. (1988). Science *241*, 202–205.

Stein, B., Baldwin, A. S. J., Ballard, D. W., Greene, W. C., Angel, P., and Herrlich, P. (1993a). EMBO J. *12*, 3879–3891.

Stein, B., Cogswell, P. C., and Baldwin, A. S. J. (1993b). Mol. Cell. Biol. *13*, 3964–3974.