Sequence and Characterization of a Coactivator for the Steroid Hormone Receptor Superfamily

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A yeast two-hybrid system was used to identify a protein that interacts with and enhances the human progesterone receptor (hPR) transcriptional activity without altering the basal activity of the promoter. Because the protein stimulated transactivation of all the steroid receptors tested, it has been termed steroid receptor coactivator–1 (SRC-1). Coexpression of SRC-1 reversed the ability of the estrogen receptor to squelch activation by hPR. Also, the amino terminal truncated form of SRC-1 acted as a dominant-negative repressor. Together, these results indicate that SRC-1 encodes a coactivator that is required for full transcriptional activity of the steroid receptor superfamily.

Steroid receptors belong to a superfamily of ligand-inducible transcription factors that regulate hormone-responsive genes. They seem to directly alter the rate of transcription by affecting the stability of the preinitiation complex (*1*). However, the finding that squelching occurs between members of the steroid receptor superfamily supports the concept that an additional factor or factors is also important for efficient ligand-inducible target gene expression (*2*).

To gain a better understanding of the mechanism of steroid receptor action, we isolated and characterized a complementary DNA (cDNA) encoding a protein that interacts and enhances hPR transactivation function. The region of hPR that encompasses the hinge and the ligand-binding domain (PRLBD, amino acids 631 to 933) was used as a bait to isolate cDNAs encoding proteins that specifically interact with hPR in the yeast two-hybrid system. From seven isolated cDNAs, we chose for further study the one that exhibited the strongest interaction with the ligand-bound PRLBD, SRC-1(.8) (Fig. 1A) (*3*). Interaction in yeast cells was observed only when PRLBD was coexpressed with SRC-1(.8) cDNA fused to the Gal4p activation domain. Neither SRC-1(.8) nor PRLBD fusion proteins were active when expressed alone. This may be due to the use of a reporter with a single and chromosomally integrated Gal4p-binding site (*5*). Snf1p and Snf4p (Fig. 1A) were used as positive controls for interaction in the two-hybrid system. Next, we examined whether the interaction of SRC-1(.8) with hPR was hormone-dependent and found that in vivo, the interaction between receptor and SRC-1(.8) is agonist-specific (Fig. 1A). Neither progesterone nor RU-486 had any effect on the activity of PRLBD or SRC-1(.8) expressed alone or on the Snf1p and Snf4p controls.

To document further that the ligand-dependent interaction observed in intact cells is due to a direct interaction with the receptor protein, we used an in vitro binding assay in which the affinity matrix was a glutathione-S-transferase (GST) fusion protein [GST fused to the A form of hPR (hPR_A)] linked to GST-Sepharose beads (Fig. 1B) (*5*). In vitro–transcribed and –translated [^{35}S]SRC-1(.8) was retained by the GST-hPR_A affinity columns when the receptor was bound to progesterone (Fig. 1B). In contrast, significantly reduced interactions were observed with ligand-free or RU-486–bound receptor, and little or no binding was observed on the GST protein column without hPR_A (*6*). Thus, interaction of SRC-1(.8) with hPR occurred in vivo and in vitro in a ligand-dependent manner.

Northern (RNA) blot analysis with polyadenylated [poly(A)$^+$] RNA from human tissues and cell lines indicated that SRC-1 is expressed as two mRNAs of ~5.5 and 7.5 kb in a variety of tissues (*6*). Conventional screening (*7*) revealed a 5.6-kb cDNA containing an open reading frame of 1061 amino acids (Fig. 2) with an apparent molecular size of 125 kD (from SDS-PAGE). The codon ATG identified here, although it generates a functional protein, may not be the authentic initiation codon. SRC-1 sequence comparisons at the amino acid level showed no significant homology to any known protein. A notable glutamine-rich region (31.4% Gln) was observed between residues 673 and 758. The NH_2-terminus of SRC-1 (residues 258 to 350) is serine- and threonine-rich (*8*).

Transient transfection assays were carried out in mammalian cell lines to further investigate the role of SRC-1 in receptor transactivation of target gene expression. HeLa cells were cotransfected with mammalian expression vectors for SRC-1 and hPR_B. Ligand-free hPR has minimal activity on the reporter either in the absence or in the presence of SRC-1 (Fig. 3A). Addition of the progesterone agonist R5020 resulted in a ~fivefold increase of hPR activity. However, when SRC-1 cDNA was coexpressed with hPR, the activity of hPR was increased an additional factor of 14.0 ± 3.3 (mean ± SEM, n = 7) . To confirm that the stimulation observed by coexpression of SRC-1 with hPR was solely dependent on the receptor and not due to an indirect effect on the basal activity of the reporter, we replaced the progesterone response element (PRE) in the reporter construct with an estrogen response element (ERE). In the absence of a DNA binding site for hPR, there was no effect of SRC-1 on the reporter construct activity, either in the absence or in the presence of the ligand (Fig. 3A). These results indicate that SRC-1 enhancement of hPR transactivation occurs through a ligand-bound receptor.

We also investigated the effect of SRC-1 on transactivation of hPR bound to the antagonist RU-486. Again, SRC-1 enhanced hPR activity in the presence of the agonist R5020 (Fig. 3B). As expected, addition of RU-486 to intact cells prevented the hormone-induced transactivation of

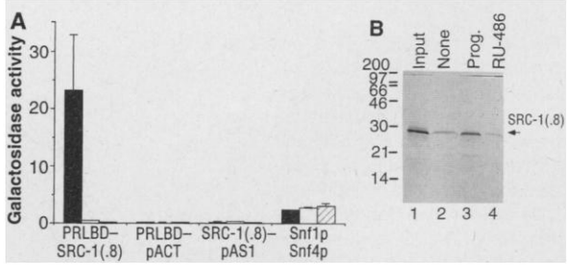

Fig. 1. Isolation of proteins that interact with hPR in a ligand-dependent manner. (**A**) Galactosidase activity of the PRLBD-interacting protein SRC-1(.8) fused to the Gal4p activation domain [PRLBD–SRC-1(.8)] in the Y190 yeast strain. The activity of PRLBD–pACT (bait construct), SRC-1(.8)–pAS1 (cDNA alone), and the positive controls Snf1p and Snf4p are shown. Yeast cells were grown in selective media containing 10^{-6} M progesterone (solid bars), no hormone (open bars), or 10^{-6} M RU-486 (hatched bars). The values represent the mean ± SEM of three independent transformations. (**B**) Interaction of SRC-1(.8) with hPR in vitro. SRC-1(.8) radiolabeled with [^{35}S]methionine (lane 1) was incubated in batch with purified baculovirus-expressed GST-hPR_A fusion protein bound to GST-Sepharose beads either in the absence (lane 2) or in the presence of 10^{-6} M progesterone (lane 3) or RU-486 (lane 4). Bound SRC-1(.8) was then eluted and analyzed in a 15% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to fluorography for ^{35}S (*5*). The input lane represents 10% of the total volume of the crude lysate used in each reaction. The numbers at the left indicate molecular size markers in kilodaltons.

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hPR. More importantly, coexpression of SRC-1 with the antagonist-bound receptor was unable to recover to any extent the RU-486–antagonized receptor transcriptional activity. This result is consistent with our observations that SRC-1 does not interact efficiently with hPR in the presence of an antagonist (Fig. 1). We and others have suggested that the antagonist RU-486 induces a distinct conformational change in the receptor molecule that impairs the ability of the receptor to transactivate (*9, 10*). Our current findings substantiate that the proper conformational change induced by an agonist is necessary for the receptor to interact with its coactivator, SRC-1. We postulate that the inability of the antagonist-bound receptor to interact efficiently with SRC-1 leads to the in vivo biological consequence of hormonal antagonism.

Next, we examined the effect of SRC-1 expression on the transcriptional activity of several intracellular receptors. SRC-1 enhanced hPR, as well as estrogen receptor (ER), glucocorticoid receptor (GR), thyroid hormone receptor (TR), and retinoid X receptor (RXR) transcriptional activity through their cognate DNA response elements (Fig. 3C). Once again, SRC-1 had little effect on the receptor activity in the absence of hormone (*6*). The spectrum of action of SRC-1 was examined through its effect on unrelated transactivators (Fig. 3D). We observed that SRC-1 enhanced the transcriptional activity of Gal4-VP16 chimeric protein; it also enhanced Sp1 transcriptional activity, albeit to a lesser extent. Notably, SRC-1 did not alter the transcriptional activity of other nuclear factors, such as E2F and E47 (*6*). Finally, we tested the influence of SRC-1 on the transcriptional activity of another inducible transcriptional factor, CREB (*11*). CREB activity was increased by the addition of forskolin to intact cells (Fig. 3D). Coexpression of SRC-1 did not affect either basal or forskolin-stimulated transcriptional activities of CREB. Therefore, although SRC-1 enhances the activity of all members of the steroid receptor superfamily as well as a few other transcriptional factors, it is not a general coactivator for all classes of transactivators.

We used a more stringent assay for coactivators that exploits the property of excess activator to sequester a limited pool of nuclear coactivator (*12*). We asked whether the coexpression of SRC-1 was able to reverse this squelching. The hormone-induced transcriptional activity mediated by hPR was reduced ~95% upon coexpression of ligand-bound hER (Fig. 4A). Addition of SRC-1 reversed this squelching by as much as ~16-fold (compare lanes 3 and 7) in a dose-dependent manner (Fig. 4A). This increase could not be explained by the enhancement of residual hPR that remains unsquelched during the assay because a ~fivefold stimulation of hPR transactivation was observed at the highest concentration of SRC-1 (lanes 2 and 8), as compared with a 16-fold recovery in the presence of SRC-1 (compare lanes 3 and 7). Such quantitative differences in activation argue strongly for reversal of squelching. We conclude that SRC-1 is a limiting factor necessary for efficient PR and ER transactivation.

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   1 MSIPRVNPSVNPSISPAHGVARSSTLPPSNSNMVSTRINR
  41 QQSSDLHSSSHSNSSNSQGSFGCSPGSQIVANVALNKGQA
  81 SSQSSKPSLNLNNPPMEGTGISLAQFMSPRRQVTSGLATR
 121 PRMPNNSFPPNISTLSSPVGMTSSACNNNNRSYSNIPVTS
 161 LQGMNEGPNNSVGFSASSPVLRQMSSQNSPSRLNIQPAKA
 201 ESKDNKEIASTLNEMIQSDNSSSDGKPLDSGLLHNNDRLS
 241 DGDSKYSQTSHKLVQLLTTTAEQQLRHADIDTSCKDVLSC
 281 TGTSNSASANSSGGSCPSSHSSLTARHKILHRLLQEGSPS
 321 DITTLSVEPDKKDSASTSVSVTGQVQGNSSIKLELDASKK
 361 KESKDHQLLRYLLDKDEKDLRSTPNLSLDDVKVKVEKKEQ
 401 MDPCNTNPTPMTKATPEEIKLEAQSQFTADLDQFDQLLPT
 441 LEKAAQLPGLCETDRMDGAVTSVTIKSEILPASLQSATAR
 481 PTSRLNRLPELELEAIDNQFGQPGTGDQIPWTNNTVTAIN
 521 QSKSEDQCISSQLDELLCPPTTVEGRNDEKALLEQLVSFL
 561 SGKDETELAELDRALGIDKLVQGGGLDVLSERFPPQQATP
 601 PLIMEERPNLYSQPYSSPFPTANLPSPFQGMVRQKPSLGT
 641 MPVQVTPPRGAFSPGMGMQPRQTLNRPPAAPNQLRLQLQQ
 681 RLQGQQQLIHQNRQAILNQFAATAPVGINMRSGMQQQITP
 721 QPPLNAQMLAQRQRELYSQQHRQRQLIQQQRAMLMRQQSF
 761 GNNLPPSSGLPVQTGNPRLPQGAPQQFPYPPNYGTNPGTP
 801 PASTSPFSQLAANPEASLANRNSMVSRGMTGNIGGQFGTG
 841 INPQMQQNVFQYPGAGMVPQGEANFAPSLSPGSSMVPMPI
 881 PPPQSSLLQQTPPASGYQSPDMKAWQQGAIGNNNVFSQAV
 921 QNQPTPAQPGVYNNMSITVSMAGGNTNVQNMNPMMAQMQM
 961 SSLQMPGMNTVCPEQINDPALRHTGLYCNQLSSTDLLKTE
1001 ADGTQQVQQVQVFADVQCTVNLVGGDPYLNQPGPLGTQKP
1041 TSGPQTPQAQQKSLLQQLLTE*
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Fig. 2. Predicted amino acid sequence of SRC-1. The numbers to the left correspond to amino acid numbers. The underlined methionine at the beginning of the sequence is the putative translation start site. The regions of the protein rich in S and T (underlined) and Q (underlined with dots) residues and the stop codon (*) are also indicated. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

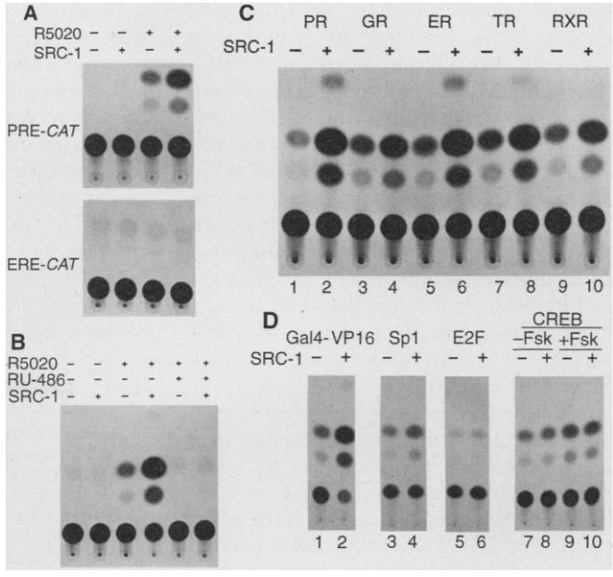

Fig. 3. (**A**) SRC-1 enhances receptor-mediated transactivation without altering basal promoter activity. HeLa cells were transiently transfected with 5 μg of PRE2-TATA-*CAT* (upper panel) or ERE-TATA-*CAT* (lower panel) reporter plasmid (*CAT*, gene for chloramphenicol acetyltransferase) along with 0.5 μg of hPR_B mammalian expression plasmid and 3 μg of SRC-1 (+) or empty expression vector (−), either in the absence (−) or in the presence (+) of 2×10^{-8} M progesterone agonist R5020. (**B**) SRC-1 does not alter the hPR basal activity when bound to the antagonist RU-486. HeLa cells were transfected as in (A) in the absence or in the presence of 2×10^{-8} M R5020 alone or in combination with 4×10^{-8} M RU-486 and then assayed for CAT activity. (**C**) SRC-1 is a general coactivator for steroid receptors. HeLa cells were cotransfected with the various steroid receptors and their cognate hormone response elements (HREs) containing reporters (*tk*, thymidine kinase promoter) [(receptor/HRE): PR/PRE2-TATA-*CAT* (lanes 1 and 2), GR/PRE2-TATA-*CAT* (lanes 3 and 4), ER/ERE2-TATA-*CAT* (lanes 5 and 6), TR/DR4-*tk*-*CAT* (lanes 7 and 8), and RXR/DR1-*tk*-*CAT* (lanes 9 and 10)] along with SRC-1 (+) or empty expression vector (−) and then treated with their ligands (PR, R5020; ER, estradiol; GR, dexamethasone; TR,Triac; and RXR, 9-*cis*-retinoic acid) at 2×10^{-8} M, and CAT activity was quantitatively determined. (**D**) Effect of SRC-1 on other transcription factors. HeLa cells were transfected with the various activators and reporters (activator/reporter), including the chimera Gal4-VP16/UASG-*tk*-*CAT* (lanes 1 and 2), Sp1/Sp1-*tk*-*CAT* (lanes 3 and 4), E2F/E2F-*tk*-*CAT* (lanes 5 and 6), and CREB/CRE-*tk*-*CAT* in the absence (lanes 7 and 8) and in the presence (lanes 9 and 10) of 1 μM forskolin (Fsk) along with SRC-1 (+) or empty expression vector (−). Because it is likely that different mammalian cells contain various concentrations of SRC-1, the choice of cell line and the precise concentration of receptor, coactivator, and reporter cDNAs used in transfection assays are of quantitative importance. The mammalian expression vectors and their cognate reporters have been described (*11, 21–24*). Lipofectin (Gibco BRL) was selected for transfection (*22*). The reporter CAT activity was determined with 100 μCi of [^{14}C]chloramphenicol and 4 mM acetyl coenzyme A as substrate and evaluated by determining the percentage of conversion of [^{14}C]chloramphenicol to the mono- and diacetylated forms (*25*).

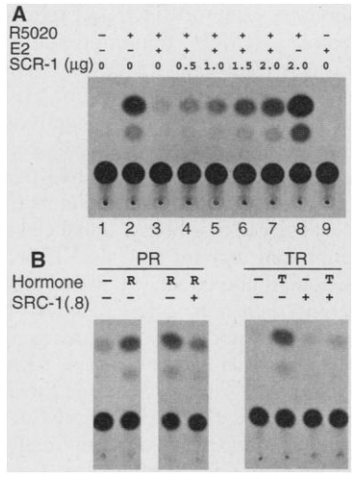

Fig. 4. (**A**) SRC-1 reverses the transcriptional interference between hPR and hER. HeLa cells were transfected with 1 μg of both hPR and hER expression vectors, together with 5 μg of PRE2-TATA-*CAT* (lanes 1 to 9) and increasing microgram amounts of SRC-1, as indicated. Cells were then exposed to ligands R5020 or E2 (2×10^{-8} M) or both, and CAT activities were determined 42 hours later from 40 μg of protein extract. The percentage of chloramphenicol conversion to the mono- and diacetylated form in lane 1 (0.11 ± 0.04), lane 2 (8.80 ± 1.05), lane 3 (0.46 ± 0.03), lane 4 (1.21 ± 0.11), lane 5 (1.72 ± 0.27), lane 6 (3.79 ± 0.86), lane 7 (7.26 ± 0.57), lane 8 (45.63 ± 7.28), and lane 9 (0.31 ± 0.04) was determined as in Fig. 3 and represents the mean ± SEM of three independent experiments. (**B**) Dominant-negative activity of NH_2-terminal truncated SRC-1. Lmtk$^-$ cells were transfected with polybrene with 5 μg of steroid receptor cDNAs expression vectors and their reporters, PR/PRE2-*tk-CAT* (PR) and TRβ/DR4-*tk-CAT* (TR), along with 10 μg of SRC-1(.8) (+) or empty expression vector (−) and then treated with hormones (R, R5020; T, thyroid hormone) (2×10^{-8} M) as indicated (*26*). The reporter CAT activity was determined with 60 μg of protein extract as in Fig. 3. SRC-1(.8) (amino acids 865 to 1061) was constructed by subcloning the Bam HI–Bgl II fragment of SRC-1(.8) into the Bgl II–Bam HI site of the PABΔgal mammalian expression vector (*24*).

We next examined whether the NH_2-terminal truncated form of SRC-1 [SRC-1(.8)], which contains the region that binds to the receptor, was able to serve as a dominant-negative inhibitor for endogenous SRC-1 function. The coexpression of SRC-1(.8) inhibited the hormone-induced transcriptional activity of hPR as well as the ligand-induced transcriptional activity of TR in Lmtk$^-$ cells (Fig. 4B). No major effect on the hormone-free receptor was observed. SRC-1(.8) also interfered with hPR transcriptional activity in HeLa and CV1 cells to a similar extent (6). The capability of the truncated SRC-1 to act as a dominant-negative repressor suggests that it is a genuine coactivator for steroid receptor target gene expression.

Multiple factors have been demonstrated to interact with steroid receptors in a ligand-dependent manner. These include a mouse bromodomain-containing protein, TIF1 (*13*), the human homolog of the adaptor Sug1p; TRIP1 (*14*) and other thyroid hormone receptor–interacting proteins (Trips) (*15*); and the ER-associated proteins ERAP160 (*16*), RIP160, and RIP80 (*17*). However, none of these proteins has been shown to enhance receptor-mediated transcriptional activity; therefore, their role as potential coactivators has yet to be defined. Recently, Cavailles *et al.* (*18*) identified RIP140 as a potential coactivator for ER. However, sequence alignment at the amino acid level reveals no significant homology between these two proteins. In addition, RIP140 has only a modest effect on ER transactivation. SRC-1 also shares no significant homology to either TIF1 or TRIP1, nor does it contain similar functional canonical domains. Therefore, it is likely that the mechanism by which SRC-1 alters the rate of transcription will differ.

Although we cannot rule out potential effects on chromatin structure, our evidence indicates that SRC-1 acts by direct contact with the receptor protein to modulate its activity. This interaction is likely to be a key regulatory event in the multiple-step steroid receptor transactivation pathway that occurs in vivo. Identification of several proteins with potential roles in steroid receptor action indicates that the activation process for steroid receptors is exceedingly complex and that the ultimate mechanism by which these factors act to modulate the transcription of specific gene networks remains to be elucidated.

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3. The coding sequence from amino acids 631 to 933 (PRLBD) of the B form of hPR, designated hPR_B, was amplified by polymerase chain reaction (PCR) with the primers 5′-CGCCATGGTCCTTGGAGGT-3′ (upper strand) and 5′-AAGTCGACACATTCACTTTTTATGAAAGAGAAG-3′ (lower strand) and cloned into the Nco I–Sal I site of the pAS1 yeast expression plasmid to be fused to the sequence for the Gal4p DNA binding domain (DBD) (*4*). Ligand-binding assays revealed that the fusion protein was expressed at 0.93 ± 0.12 pmol/mg of protein (mean ± SE, n = 7). For the two-hybrid screening, the *Saccharomyces cerevisiae* strain Y190 containing the Gal4DBD-PRLBD expression plasmid (pAS1-Cyh) was transformed with a human β lymphocyte cDNA expression library, and the transformants were screened for interacting proteins in the presence of 10^{-6} M progesterone. The specificity of the interacting proteins was assessed by mating Y190 cells containing the SRC-1(.8) cDNA with the Y187 strain containing pAS1-Snf, pAS1-p53, pAS1-Cdk, and pAS1-lamin (*4*).
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5. For in vitro transcription and translation, the SRC-1(.8) cDNA Xho I fragment was cloned into the Sal I site of the pT7BSall vector (*10*). Receptor-specific affinity resins were constructed by linking recombinant baculovirus glutathione-S-transferase hPR_A (GST-hPR_A) to GST-Sepharose beads as described (*19*). Receptors were activated in vivo by the addition of 10^{-8} M hormones (progesterone or RU-486) to intact cells 24 hours before harvesting. Whole-cell extracts were treated for an additional 15 min at 30°C with 10^{-6} M hormone before purification. Approximately 400 μg of total protein extract was incubated with 20 μl of GST-Sepharose beads in suspension (Pharmacia) for 2 hours at 4°C. Resins were then washed twice with NENT buffer [20 mM tris-OH (pH 8.0) containing 100 mM NaCl, 1 mM EDTA, 0.5% NP-40, and 0.5% milk powder] and washed twice more with transcription buffer [20 mM Hepes (pH 7.9) containing 60 mM NaCl, 1 mM dithiothreitol, 6 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol]. Subsequently, the beads were mixed with 25 μl of in vitro–transcribed and –translated [^{35}S]methionine-labeled SRC-1(.8) crude lysate (Promega), and interactions were allowed to occur at 4°C for 1 hour. Bound proteins were eluted with 0.2% SDS in 10 mM Tris-OH buffer (pH 7.6), fractionated on SDS-PAGE, and subjected to fluorography for ^{35}S.
6. S. A. Oñate, S. Y. Tsai, M.-J. Tsai, B. W. O'Malley, data not shown.
7. After the identification of SRC-1(.8), the Xho I insert cDNA was used to screen a fibroblast library constructed in λZAP (*20*). Two clones, 154-22a of 1.4 kb and 154-25 of 2.3 kb, encompassed ~3.6 kb of the most 3′ end of SRC-1. The clone 154-22a also contained an additional 0.8 kb of 5′ sequence not related to SRC-1. The remaining 5′ end sequence of SRC-1 was cloned by PCR amplification from a λgt11 HeLa library (Clontech) by using 10^9 phages with the λgt11 forward primer and the nested primer 5′-GGAATTCCCGACGTTGTGCCAACA-3′. First, amplification was performed for 1 min at 94°C, 1 min at 72°C, and 2 min at 72°C, then followed for another five cycles with a progressive decrease in the annealing temperature of 1°C per cycle, from 71° to 67°C. Then, amplification continued for 29 cycles (1 min at 94°C, 1 min at 64°C, 2 min at 72°C) with a final extension of 5 min at 72°C. The PCR products were cloned and sequenced. Amplified cDNAs containing identical sequence to the 5′ end of clone 154-22a were assigned as positives. The Eco RI (partial) and Bsm I insert from the longest cDNA amplified (2.2 kb) was ligated to the Bsm I–Sal I insert from 154-22a, then religated into the Eco RI–Xho I sites of the mammalian expression vector pBK-CMV (Strategene), and renamed SRC-1 (accession number U40396). The 3′ end untranslated region of SRC-1 from clone 154-25 (2.3 kb) was not included.
8. Current sequence comparisons with the BLAST algorithm show that amino acids 605 to 1005 are identical to the *hin2* gene. This gene was identified by analysis of a human immunodeficiency virus–type 1 promoter insertion in vivo (accession number U19179) and its meaning is unclear. In addition, partial DNA sequences for SRC-1 have been isolated randomly (accession numbers T56159 and U19179). No function for these partial cDNAs has been described.
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27. We would like to thank S. Law for his assistance in sequencing SRC-1 and P. Samora for his technical assistance. We also thank S. Elledge for providing the materials for the yeast two-hybrid system and M. Montminy for providing CREB expression vector and CREB response DNA element. This work was supported by NIH grant HD08188 and American Cancer Society grant BE63.

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Self-Release of CLIP in Peptide Loading of HLA-DR Molecules

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The assembly and transport of major histocompatibility complex (MHC) class II molecules require interaction with the invariant chain. A fragment of the invariant chain, CLIP, occupies the peptide-binding groove of the class II molecule. At endosomal pH, the binding of CLIP to human MHC class II HLA-DR molecules was counteracted by its amino-terminal segment (residues 81 to 89), which facilitated rapid release. The CLIP(81–89) fragment also catalyzed the release of CLIP(90–105) and a subset of other self-peptides, probably by transient interaction with an effector site outside the groove. Thus, CLIP may facilitate peptide loading through an allosteric release mechanism.

The peptide-binding groove of MHC class II molecules is blocked in the endoplasmic reticulum by association with the invariant chain (Ii) (*1*). Proteolytic digestion of Ii in endosomal compartments generates CLIP (*2*), which encompasses the region of Ii involved in binding to MHC class II (*3*) and must be released from class II molecules before loading with peptide (*4*). The nonclassical MHC class II molecule HLA-DM has been implicated in the removal of CLIP from class II molecules (*5*, *6*). However, class II alleles differ with regard to their dependency on HLA-DM. Thus, for example, efficient removal of CLIP from DR3 and DR11 in vivo and subsequent peptide loading depend on the expression of HLA-DM (*4*), whereas no comparable requirement for HLA-DM is apparent for DR4Dw4, A^d, or A^k (*4*, *7*). In addition, loading with conventional self-peptides other than CLIP has been demonstrated in most DR^+DM^- mutant cells, with the proportion of non-CLIP peptides varying between different class II alleles (*2*–*4*). These observations suggest that CLIP removal does not depend absolutely on HLA-DM, even for alleles, such as A^d, that bind CLIP with high affinity (*8*, *9*).

The peptide repertoire of DR3 molecules from the DM-negative mutant cell line T2.DR3 was assessed by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS). The repertoire was characterized by a clustering of signals with mass-to-charge ratios (m/z) of 1500 to 2300, indicative of conventional self-peptides, that accounted for ~30 to 50% of the total peptides in various DR3 preparations (Fig. 1). The remaining 50 to 70% of the peptides were accounted for by four prominent CLIP variants. In general, a smaller proportion of CLIP peptides (10 to 20%) is associated with DR3 from DM-positive, wild-type cells.

A possible explanation for the partial peptide loading of DR3 in DM-negative T2.DR3 mutants is that CLIP may facilitate its own dissociation from the class II molecule in the absence of HLA-DM. To test this hypothesis, we determined the kinetics of CLIP release from DR3 by performing in vitro peptide binding assays based on high-performance size-exclusion chromatography (HPSEC) (*10*). An important methodological parameter that influences CLIP dissociation is the detergent used (*4*). In a detergent-free system, labeled CLIP(81–105) dissociated rapidly from recombinant soluble DR3 and DR1 (sDR3 and sDR1) produced in insect cells, displaying a time of half-maximal dissociation, $t_{1/2}$, of ~2 hours at 37°C and pH 5.8 (Fig. 2A). NP-40, a detergent often used in binding assays involving CLIP, prevented rapid dissociation of CLIP, thereby explaining the slow rate of dissociation observed in other studies (*6*, *9*, *11*). In contrast, the detergent Zwittergent-12 (ZW-12) slightly increased the rate of CLIP dissociation. The high rate of CLIP dissociation was not a peculiarity of recombinant DR molecules; similar rates ($t_{1/2}$ of ~30 min in the presence of ZW-12) were observed with sDR3 and with DR3 from T2.DR3 cells (Fig. 2B). The rate of CLIP dissociation increased further at pH 4.8 ($t_{1/2} \approx 15$ min), whereas the antigenic peptide HSP65(3–13) remained stably bound at this pH ($t_{1/2} > 48$ hours) (Fig. 2B). Dissociation of endogenously bound CLIP variants from DR3 molecules isolated from T2.DR3 cells and depleted of detergent showed a time course similar to that for the sDR3:CLIP complexes generated in vitro ($t_{1/2} \approx 1.5$ hours), as revealed by MALDI-MS kinetics. Thus, DR3:CLIP complexes formed in vivo and in vitro display comparably low stabilities in the absence of detergent (*12*).

The COOH-terminal region of CLIP is thought to occupy the peptide-binding groove of MHC class II molecules in a manner similar to antigenic peptides (*9*, *11*), with the NH_2-terminal residues protruding from the groove. Both NH_2- and COOH-terminal extensions of antigenic peptides usually confer increased stability to MHC class II αβ:peptide complexes during SDS–polyacrylamide gel electrophoresis (*13*). However, long CLIP peptides containing NH_2-terminal extensions are released relatively rapidly from DR molecules

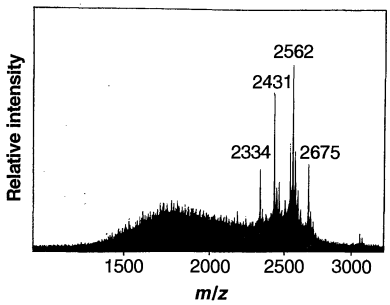

Fig. 1. Mass profile of DR3-associated self-peptides from T2.DR3 cells: Endogenous peptides released by acid treatment of 5 μg of affinity-purified DR3 molecules (*24*) from the human DM-negative mutant Epstein-Barr virus–transformed B/T hybrid cell line T2.DR3 (T2 cells transfected with DR3) were analyzed by MALDI-MS (*25*). The indicated masses correspond to CLIP(82–102) (m/z = 2334), CLIP(82–103) (m/z = 2431), CLIP(82–104) (m/z = 2562), and CLIP(81–104) (m/z = 2675) (*3*).

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